STRUCTURE AND FUNCTION STUDIES OF SUCCINATE-UBIQUINONE REDUCTASE FROM *ESCHERICHIA COLI*

By

XUDONG YANG

Bachelor of Science

Nankai University

Tianjin, P.R.China

1993

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
December, 1998

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Thesis Approved:

Thesis Advisor

Dean of the Graduate College

ACKNOWLEDGEMENTS

I wish to express sincere gratitude to my advisor Dr. Chang-An Yu for his guidance, support and encouragement throughout my graduate program. My sincere appreciation extends to my graduate committee members: Dr. Linda Yu, Dr. Robert Matts, Dr. Olin Spivey, and Dr. Mario Rivera, for their suggestions and assistance.

I would also like to thank Dr. Richard Essenberg for his suggestions and assistance, Dr. Roger Koeppe for the critical review of the manuscripts, Dr. Dayan He and Dr. Anatoly Kachurin for their help in this study.

Finally I would like to deeply thank my wife, Hua Tian, for her support and patience, and my parents, Yougeng and Minglan, for their inspiration and encouragement.

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ABBREVIATIONS

[^{3}H]azido-Q	3-azido-2-methyl-5-methoxy[^{3}H]-6-geranyl-1,4-benzoquinone
[^{3}H]-5-azido-Q	5-azido-2,3-dimethoxy[^{3}H]-6-geranyl-1,4-benzoquinone
bp	base pairs
CO	carbon monoxide
cyt	cytochrome
DCPIP	dichlorophenol-indophenol
E9C12	polyoxyethylene-9-lauryl ether
E. coli	Escherichia coli
EPR	electron paramagnetic resonance
HPLC	high performance liquid chromatography
kDa	kilodaltons
kb	kilo base pairs
PMSF	phenylmethylsulfonyl fluoride
Q	ubiquinone
Q_2	2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone
QFR	quinone-fumarate reductase
QPs	the membrane-anchoring protein fraction of bovine heart mitochondrial succinate-ubiquinone reductase
S-1, S-2, S-3	iron-sulfur centers 1, 2, 3, respectively
SDH	succinate dehydrogenase
sdhC, D, A, B	genes incoding the four subunits of *E. coli* SQR
SdhC, D, A, B	the protein form of the four subunit of *E. coli* SQR

the SdhC-SdhD fraction	the membrane-anchoring protein fraction of *E. coli* succinate-ubiquinone reductase
SQR	succinate-ubiquinone reductase
TTFA	thenoyltrifluoroacetone

CHAPTER I

INTRODUCTION

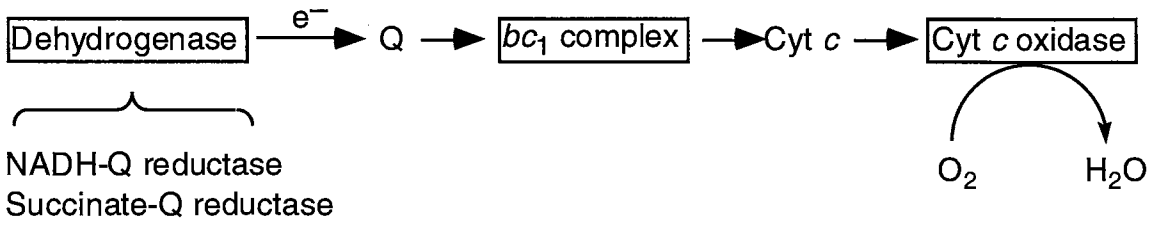
I. General

Succinate-ubiquinone reductase (SQR), also called complex II, catalyzes two electrons transfer from succinate to ubiquinone. It is a transmembrane protein and an essential segment of the Kreb cycle and mitochondrial respiratory chains. In the mitochondrial electron transport chain (Scheme I), electrons are transferred from dehydrogenases, namely NADH-Q reductase and succinate-Q reductase to the bc_1 complex through quinone. These electrons are then passed to cytochrome *c* oxidase to reduce O_2. Bacterial systems during aerobic growth (Scheme I) also contain several kinds of dehydrogenases, including NADH-Q reductase, succinate-Q reductase, and glycerol-3-P dehydrogenase etc. Electrons are directly passed to quinone oxidases (cytochrome *bo* and *bd* complex in *E. coli*) through quinone to produce H_2O.

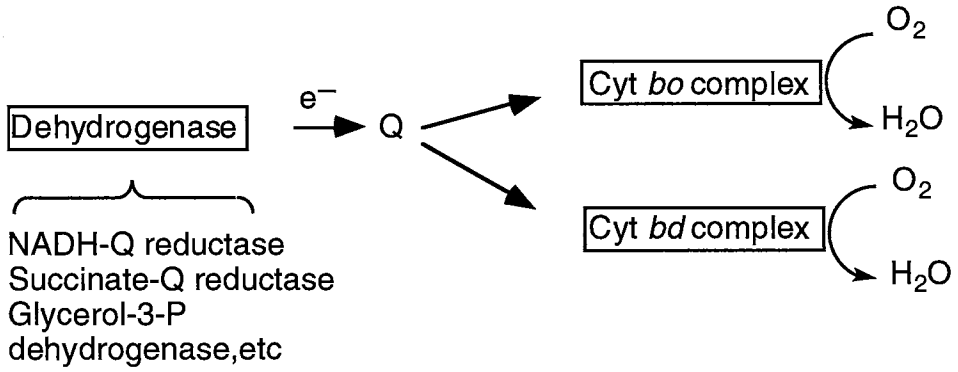
SQR contains a membrane-anchoring domain and a peripheral domain that is exposed to the cytoplasmic side of bacteria or the matrix side of mitochondria. The peripheral domain is the citric acid cycle enzyme succinate dehydrogenase (SDH) (Scheme II). The membrane-anchoring domain is generally composed of two transmembrane subunits (that from bovine mitochondria has three (1) and that from *B. subtilis* has one (2)). The transmembrane subunits have been given various names: SdhC and SdhD, CII_3 and CII_4, QPs1, QPs2, and QPs3 (3). The name QPs is used in our laboratory for Q-binding proteins. Bacteria also contain a structurally and functionally similar enzyme, the quinol:fumarate reductase (QFR), which catalyzes the opposite enzymatic reaction *in vivo*,

Scheme I. Electron transport chain components of mitochondria and bacteria (*E. coli*)

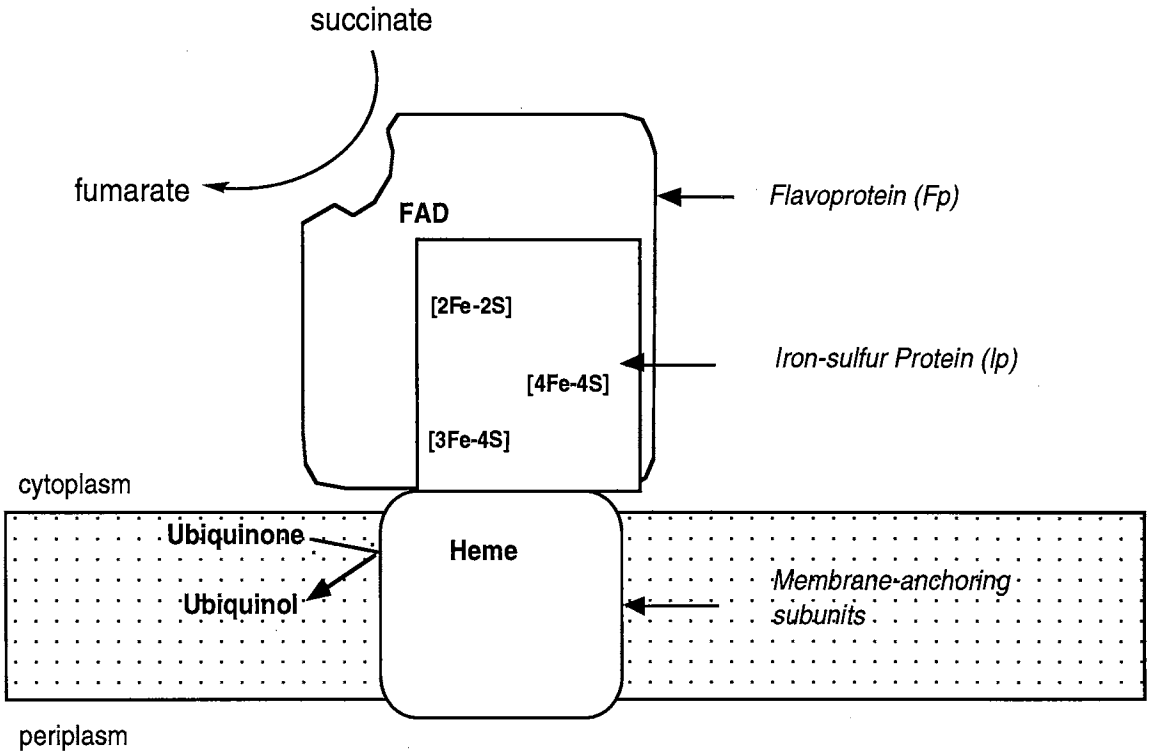
Mitochondria Electron Transport Chain



Bacterial Electron Transport Chain during Aerobic Growth (*E. coli*)



Scheme II. Schematic diagram of SQR



and is expressed during anaerobic growth using fumarate as terminal electron acceptor. SQR from various sources generally has four subunits: Flavoprotein (Fp or SdhA; 64-79 kDa), which has one covalently bound FAD; and Iron-sulfur protein (Ip or SdhB; 27-31 kDa), which has three iron-sulfur clusters: [2Fe-2S] (S1), [4Fe-4S] (S2), [3Fe-4S] (S3); and SdhC/CII_3/QPs1 (13-18 kDa), and SdhD/CII_4/QPs3 (11-16 kDa), which house one or two protoheme XI (heme b_{556}) and provide quinone-binding site (s). The protein topology and locations of the prosthetic groups can be illustrated in Fig. 1, based on studies of antibody probing, surface labeling, and electron micrographs (3).

II. The Peripheral Domain--Succinate Dehydrogenase (SDH).

The understanding of the structure and function of SQR started with the study of the soluble SDH. From different species, the amino acid sequences of the Fp and Ip subunits are highly conserved (4-6). For example, the sequences of the Ip subunits from bovine SQR and from *E. coli* are approximately 50% identical. For Fp subunits, there are 10 conserved amino acid residue stretches from different species, defined as segment a, b, c, d, e, f, g, h, i, j (3). On the other hand, amino acid sequences of membrane-anchoring subunits differ significantly among species (7-12). Of the available amino acid sequences of membrane-anchoring subunits, QPs1 from bovine mitochondria has higher sequence similarity to SdhC of *E. coli* than to SdhC of other bacteria. However, QPs2 or QPs3 from beef heart mitochondria have no sequence similarity to *E. coli* SdhD.

The two half cell reactions of SQR are:

Succinate --> fumarate + $2H^+$ + $2e^-$ (E_m = +30 mV)

Ubiquinone +$2H^+$ +$2e^-$ --> ubiquinol (E_m = –75 mV to +120 mV). SDH activity (succinate/ fumarate couple) is assayed using artificial electron acceptors, such as phenazinemethosulfate (PMS, E_m =+80 mV), and ferricyanide (+360 mV). SQR activity is assayed using dichlorophenolindophenol (DCPIP, E_m = +220 mV) as a final electron acceptor, with or without quinone analogs.

The Fp subunit of SDH contains the dicarboxylate binding site. Oxidation of succinate to fumarate is a trans-dehydrogenation (13). Malonate and oxaloacetate are potent competitive inhibitors of SDH (3). D- and L- malate are poor substrates for SDH since they are oxidized at the active site to enol-oxaloacetate that inhibits the enzyme (14). Chemical modifications in combination with mutant studies have been used to identify residues in the active site of the Fp subunit of SDH. Bovine SQR, *E. coli* SQR and QFR, and *W. succinogenes* QFR are very sensitive to thiol-modifying reagents and substrate protect these enzymes from inactivation (15). A cysteine residue in segment-g of Fp subunit was identified to be located in the active site (15). There is a conserved arginine residue in segment-g next to the cysteine. It is suggested to form a bidentate ionic pair with one of the carboxylate groups of succinate, serving to orient the substrate at the active site (16). Segment-f in Fp of SDH contains a conserved triad, H-P-T. This histidine has been suggested to assist in the deprotonation of succinate (17).

Segment-b of Fp contains the histidine residue, to which FAD is covalently bound via N(3)-8α -riboflavin linkage (18). FAD is covalently bound to Fp prior to enzyme assembly (19). In *B. subtilis* SQR mutants lacking flavin (a G-D mutation in segment-b of Fp) the iron-sulfur centers are not reduced by succinate, but can be reduced by reversed electron transfer from menaquinol in the membrane (20,21). This suggests that the flavin is probably located close to the substrate binding site and is most likely the first electron acceptor during succinate oxidation. In addition, succinate oxidation is only possible when the FAD is covalently bound. *Desulfovibrio multispirans* QFR contains non-covalently bound flavin and can not oxidize succinate (22). FAD is a two electron carrier, in contrast to the other redox prosthetic groups (iron-sulfur clusters, hemes) in SQR/QFR. FAD has a very stable free radical, which provides a mechanism to allow the two electrons from succinate oxidation to be passed to iron-sulfur protein one at a time (23).

The Ip of SDH contains three clusters of cysteine residues, which are ligands to three iron-sulfur clusters. The N-terminal domain houses the [2Fe-2S] cluster (S1), ligated

by a CxxxxCxxC.....C motif (3). The [2Fe-2S] cluster is most likely the first electron acceptor after flavin in SQRs. The C-terminal domain harbors the [4Fe-4S] (S2) and [3Fe-4S] (S3) clusters, ligated by CxxCxxCxxxCP.....CxxCxxCxxxCP motif (24,25). The [4Fe-4S] cluster generally has a very low potential and its function is not clear. In bovine SQR, it has been proposed that [4Fe-4S] and cytochrome b_{556} form a low potential electron transfer chain (26) (Scheme III). However, there is no evidence to support this hypothesis. The [3Fe-4S] cluster has an important structural role, because isolated bovine SDH can be reconstituted with QPs only when cluster S3 is protected under anaerobic conditions (27-29). It has been suggested by the following facts that cluster S3 is close to the quinone binding site, and functions as electron donor to quinone. The mammalian SQR is sensitive to both TTFA and carboxins. These two inhibitors share some common structural features (Scheme IV) and were shown to interact with the same site (30). The inhibitors prevent the reduced center S3 from being reoxidized by quinone but do not prevent S3 from being reduced by succinate (31). Addition of TTFA to heart submitochondrial particles has two impacts: the quenching of the split signals of the ubisemiquinone pair in SQR, and the shifting of the midpoint potential of S3 (32,33). A mutation in *U. maydis* SQR that conferred resistance to carboxin was located in the S3 motif (34).

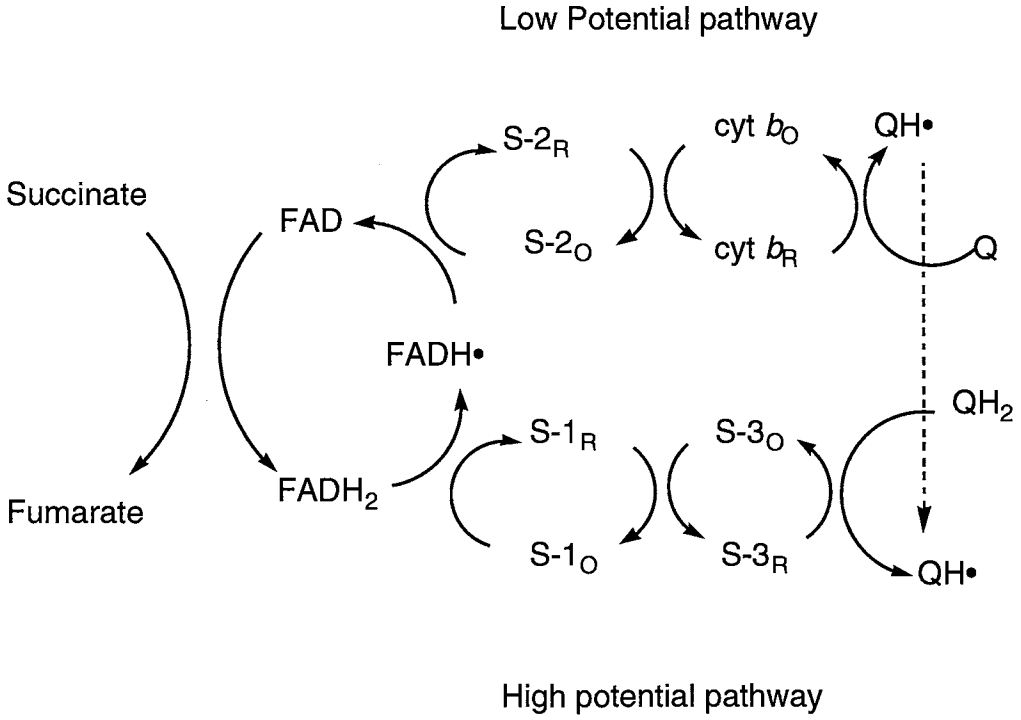
Bovine SQR has been reported to associate with bc_1 complex to form SCR (succinate-cytochrome *c* reductase) supercomplex. Under anaerobic conditions, reconstitutively active SDH (soluble) is released from SCR (insoluble) at alkaline conditions (pH 10.0). After centrifugation, the supernatant containing SDH is collected and further purified by calcium phosphate gel chromatography (35).

III. The Membrane-Anchoring Subunits.

1. Topology

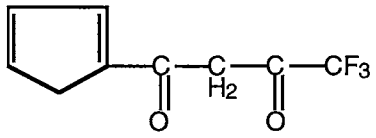
The electron transport mechanisms in SQR are still not fully understood, although the participation of FAD and iron-sulfur clusters S1 and S3 is established. In our

Scheme III. Dual pathway hypothesis of electron transfer mechanism in SQR.

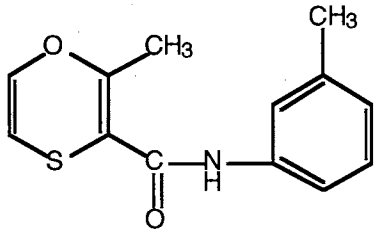


*Subscripts O and R indicate oxidized and reduced states respectively.

Scheme IV. Structures of two important SQR inhibitors



TTFA



carboxin

laboratory, we focus on the study of the membrane-anchoring subunits from bovine SQR, especially the protein:quinone interaction.

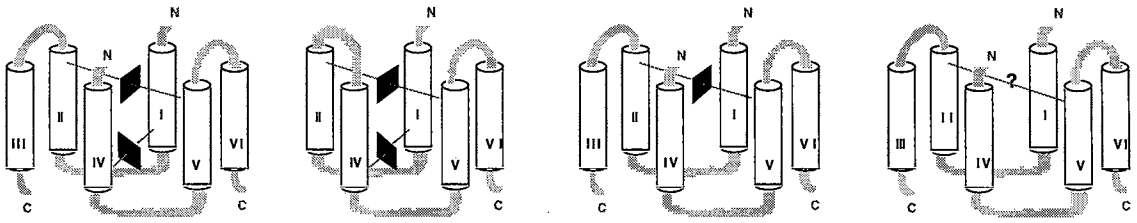
The membrane anchor consists of one larger (five transmembrane helices) or two smaller polypeptides (each has three transmembrane helices). These topologies are predicted by hydropathy plot and are confirmed by various experiments. For example, *B. subtilis* SdhC subunit is genetically fused with alkaline phosphatase at various positions (37). The system is based on the fact that *E. coli* alkaline phosphatase (PhoA) requires a N-terminal signal sequence in order to be transported from the cytoplasm to the periplasm to become active protein. When the fusion is made in the periplasm loop region of a membrane protein, the membrane protein can substitute for the lacking export signal sequence of PhoA (37). It has been shown that this SdhC contains five transmembrane helices as predicted, with the N-terminal exposed to the cytoplasm (37). The topology of QPs3 from bovine SQR was confirmed by peptide immunochemistry (38).

SQR/QFR membrane anchors have been structurally divided into four types (Scheme V), based on available sequences (39): Type A anchors have two polypeptides, with three transmembrane helices each, and contain two protoheme IX molecules (b_H and b_L). Type B anchors have one polypeptide with five transmembrane helices and contain heme b_H and heme b_L. Type C anchors have the same polypeptide arrangement as Type A, but contain only one heme, corresponding to heme b_H although its midpoint potential varies in different organisms. Type D membrane anchors have two polypeptides, with three transmembrane helices each, and are without heme.

2. The Heme *b* of the Membrane-Anchoring Subunits.

The classification of four types of membrane anchors is to account for the fact that the redox properties and heme content varied significantly among all species of SQRs, which brought much controversy to the study of their functions. *B. subtilis* SQR contains two hemes *b* , and the heme b_H (E_m = +65 mV) is reducible by succinate, while the heme b_L (E_m = -95 mV) is not (40,41). *E. coli* and *Ascaris suum* SQR have one heme *b* (b_H).

Scheme V. Schematic diagram of structure of the membrane-anchoring subunits of SQR



Type A	Type B	Type C	Type D
T. acidophilum	*B. subtilis*	*E. coli*	*S. cerevisiae*
SdhC and SdhD	SdhC	SdhC and SdhD	SdhC and SdhD

The heme *b* in *E. coli* SQR (E_m = +36 mV) is fully reducible by succinate (42), while the heme *b* in *A. suum* SQR (E_m = –34 mV) is only partially reduced by succinate (43,44). Cytochrome b_{560} in bovine SQR (E_m =–185 mV) is not reducible by succinate and is present in substoichiometric amounts, with respect to FAD (45). Furthermore, purified SQR from yeast contains no heme *b* (46).

The ligands for cytochrome *b* in *B. subtilis* (47,48), bovine (49), and *E. coli* (50) SQR have been identified as bis-histidines by magnetic circular dichroism. In *B. subtilis* enzyme, the histidine residues have been identified by site-directed mutagenesis: His-28 and His-113 are ligands for heme b_L, while His-70 and His-155 are ligands for heme b_H (37). In *E. coli* SQR, site-directed mutagenesis reveals the two histidine ligands are from SdhC (His-84) and SdhD (His-71), respectively (51). The sequence alignment reveals two conserved histidines from the membrane-anchoring subunits of SQR from all species (Fig. 1) (39).

A structural model for the membrane anchor was thus proposed by first aligning the heme axial ligands and transmembrane segments (Fig. 1). Helices I, II, IV and V, on which the histidine axial ligands to heme are distributed, form a four helix bundle in the model (Scheme V)

There are some evidences that cytochrome *b* plays a structural rather than a catalytic role.

(i). In *B. subtilis* SQR, insertion of heme into apocytochrome *b* subunits is crucial for the assembly of the membrane-bound enzyme, suggesting a structural role for the enzyme (52).

(ii). In bovine SQR, the absorption properties, carbon monoxide reactivity, redox potential, and EPR characteristics of cytochrome b_{560} in isolated QPs differ from those in intact SQR (45). These characteristics of cytochrome b_{560} were restored when QPs was reconstituted with SDH to form SQR, indicating the structural role of cytochrome b_{556} in the interaction with SDH (45).

SdhC

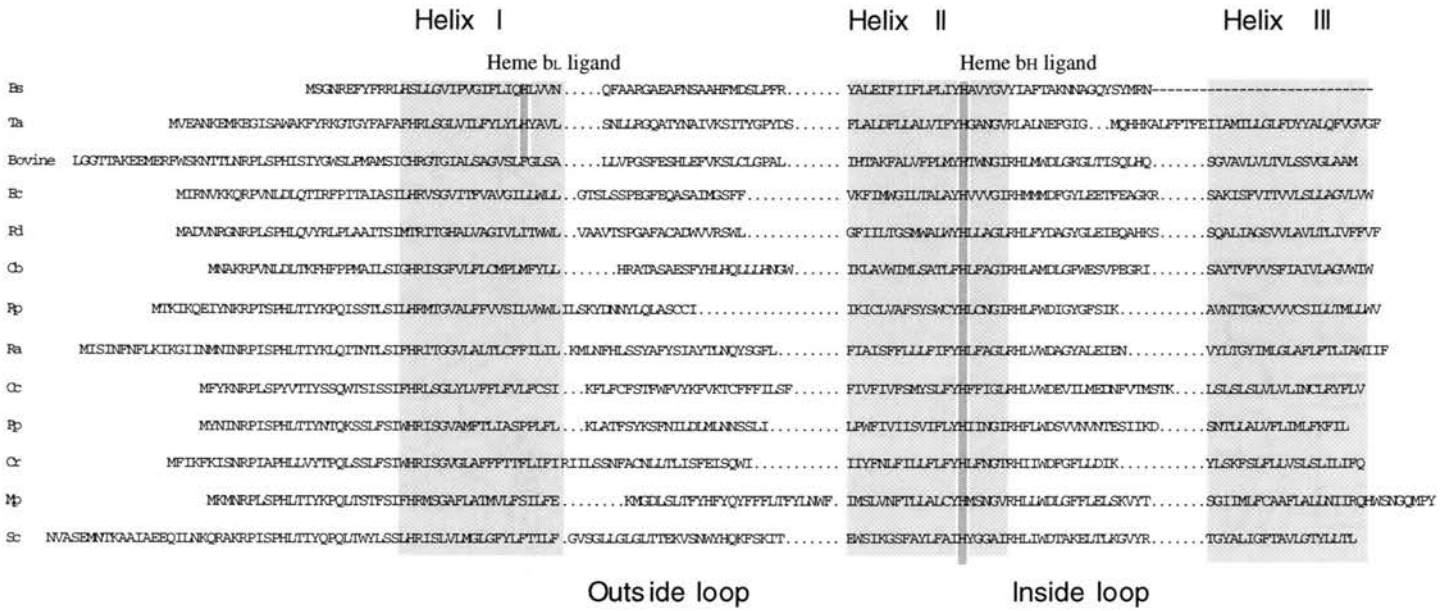
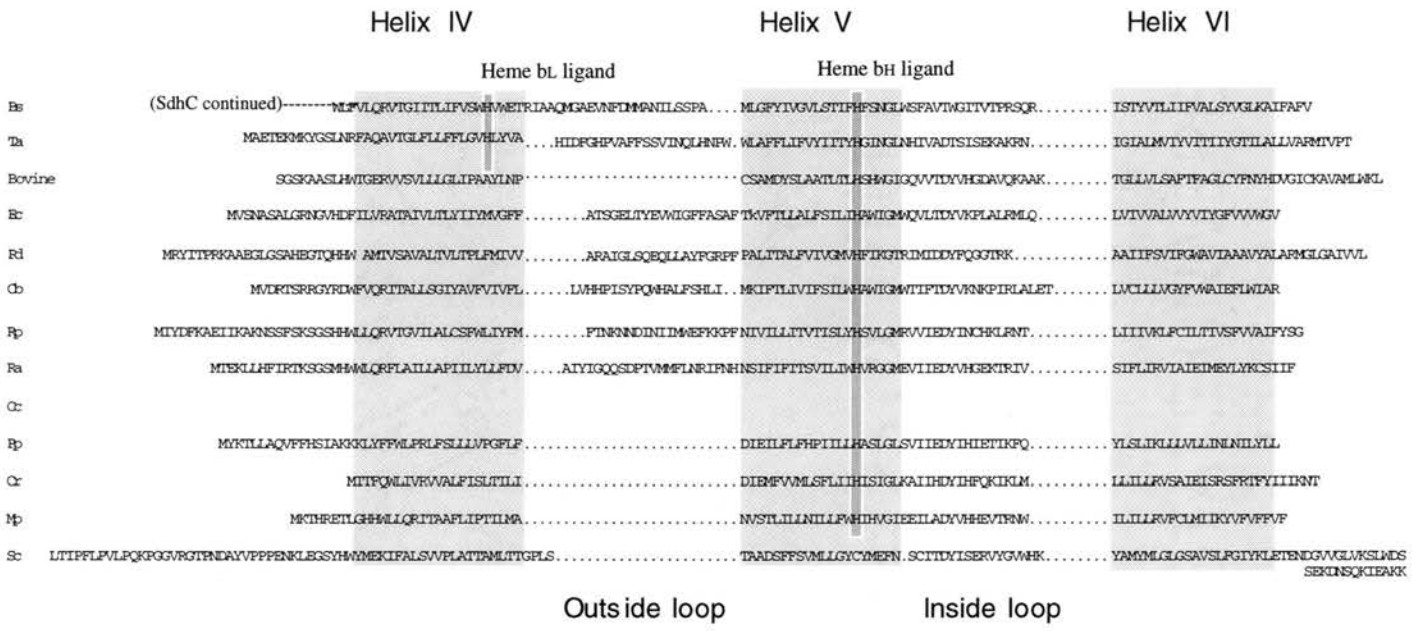


Fig. 1 Sequence comparison of SdhC and SdhD subunits. The abbreviations for the organisms are: Bs, *Bacillus subtilis*; Ta, *Thermoplasma acidophilum*; Ec, *Escherichia coli*; Pd, *Paracoccus denitrificans*; Cb, *Coxiella burnetii*; Rp, *Rickettsia prowazekii*; Ra, *Rectinomonas americana*; Ca, *Cyanidum caldarium*; Pp, *Porphyra purpurea*; Cr, *Chondrus crispus*; Mp, *Marchantia polymorpha*; Sc, *Saccharamyces cerevisiae*.

SdhD


Helix IV
Helix V
Helix VI
Heme bL ligand
Heme bH ligand
Bs (SdhC continued)--------MLPVLQRVTGIITLIFVSWHVWETRIAAQMGAEVNFDMMANILSSPA....MLGPYIVGVLSTIPHFSNGLWSFAVIWGITVIPRSQR.....ISTYVILIIFVALSYVGLKAIFAFV
Ta MAETEKMKYGSLNRFAQAVTGLFLLFFLGVHLYVA....HIDPGHPVAPFSSVINQLHNFW.WLAFFLIFVYIITYHGINGLNHIVADTSISEKAKRN.....IGIALMVIYVITIIYGTILALLVARMIVPT
Bovine SGSKAASLHWIGERVVSVLLLGLIPAAYLNP.....CSAMDYSLAATLTLHSHWGIGQVVIDYVHGDAVQKAAK.....TGLLVLSAPIFAGLCYPNYHDVGICKAVAMLWKL
Bc MVSNASALGRNGVHDFILVRATAIVLTLYIIYMVGFF.....ATSGELTYEVWIGFFASAFTKVFTLLALFSILIHAWIGMWQVLIDYVKPLALRMLQ.....LVIVVALVVYVIYGFVVVWGV
Rl MRYTTPRKAAEGLGSAHEGTQHHW AMIVSAVALIVLTPLFMIVV.....ARAIGLSQEQLLAYPGRPFPALITALFVIVGMVHFIKGTRIMIDDYPQGTRK.....AAIIPSVIRGWAVIAAAVYALARMGLGAIVVL
Cb MVDRTSRRGYRDWFVQRITALLSGIYAVFVIVPL.....LVHHPISYPQWHALFSHLI..MKIPTLIVIFSILVHAWIGMWTIFTDYVKNKPIRLALET.....LVCLLLVGYFVWAIEFLWIAR
Rp MIYDPKAEIIKAKNSSFSKSGSHHWLLQRVTGVILALCSPWLIYPM.....PTNKNNDINIIMWEFKKPFNIVILLITVTISLYHSVLGMRVVIEDYINCHKLRNT.....LIIIVKLFCILTTVSPVVAIFYSG
Ra MTEKLLHFIRTKSGSMHWWLQRFLAILLAPIILYLLFDV.....AIYIGQQSDPTVMMFLNRIFNHNSIFIFTTSVILIWHVRGGMEVIIEDYVHGEKTRIV.....SIFLIRVIAIEIMEYLYKCSIIF
Cc
Rp MYKTLLAQVFFHSIAKKKLYFFWLPRLFSLLLVPGFLF.....DIEILFLFHPIILLRASLGLSVIIEDYIHIETIKFQ.....YLSLIKLLLVLLINLNILYLL
Cr MITFQWLIVRVVALFISLITLI.....DIEMFVVMLSFLIIHISIGLKAIIHDYIHFQKIKLM.....LLILLRVSAIEISRSFKTFYIIIKNT
Mp MKTHRETLGHHWLLQRITAAFLIPTILMA.....NVSTLILLNILLFWHIHVGIEELLADVVHHEVIRNW.....ILILLRVPCLMIIKYVFVFFVF
Sc LTIPFLFVLPQKPGGVRGTPNDAVVPPPENKLEGSYHWYMEKIFALSVVFLATTAMLTTGPLS.....TAADSFFSVMLLGYCYMEFN.SCITDYISERVYGVWHK.....YAMYMLGLGSAVSLPGIYKLETENDGVVGLVKSLWDS SEKDNSQKIEAKK
Outside loop
Inside loop

(iii). In *E. coli* SQR, after replacement of the crucial histidine residues by mutagenesis, the apo-heme enzyme retains some activity (51). Moreover, the *E. coli* cytochrome b_{556} become reduced by succinate when cross-reconstituted with bovine SDH. However, no electron transfer activity is detected, suggesting that reduced cytochrome b_{556} may not pass electron to quinone (36).

3. The Quinone-Protein Interaction on the Membrane-Anchoring Subunits.

The Q-binding activity of the membrane-anchoring subunits is indicated by several lines of evidence:

(i). SDH alone does not catalyze the electron transfer from succinate to ubiquinone, although it can pass electrons to artificial electron acceptors. Addition of QPs to SDH converts SDH into SQR (53).

(ii). The detection of a ubisemiquinone radical in bovine SQR and SCR indicated the specific interaction of quinone with SQR (54,55). The ubisemiquinone radical is not detected in SDH, whereas it can be detected in the reconstituted SQR (SDH + QPs) (56). This result demonstrated that the Q-binding site is located in the QPs. The ubisemiquinone was shown to have the split signals at low temperatures arising from spin-spin interactions between two semiquinones. The ubisemiquinone radical is also detected in *E. coli* SQR by He and coworkers (unpublished result). However, the Q_s semiquinone pair has only been observed in mammalian mitochondrial SQR, some green plant mitochondria (57) and in mitochondria from *Neurospora crassa* (58), but not in a wild type bacterial SQR/QFR.

(iii). Photoaffinity labeling of bovine SQR using [^{3}H]azido-Q derivatives revealed that about 50% of the radioactivity is found in QPs1, 25% and 25% are found in QPs2 and QPs3, respectively, and no radioactivity is found in SDH (1). The Q-binding domains in QPs1 is located at the loop connecting transmembrane helices II and III which extrudes from the surface of the M-side of the mitochondrial inner membrane (10) (Fig 2). The Q-binding domain in QPs3 is located at the end of transmembrane helix I toward the C-side of the membrane (11) (Fig 2). The Q-binding domain(s) in other succinate-Q reductases has

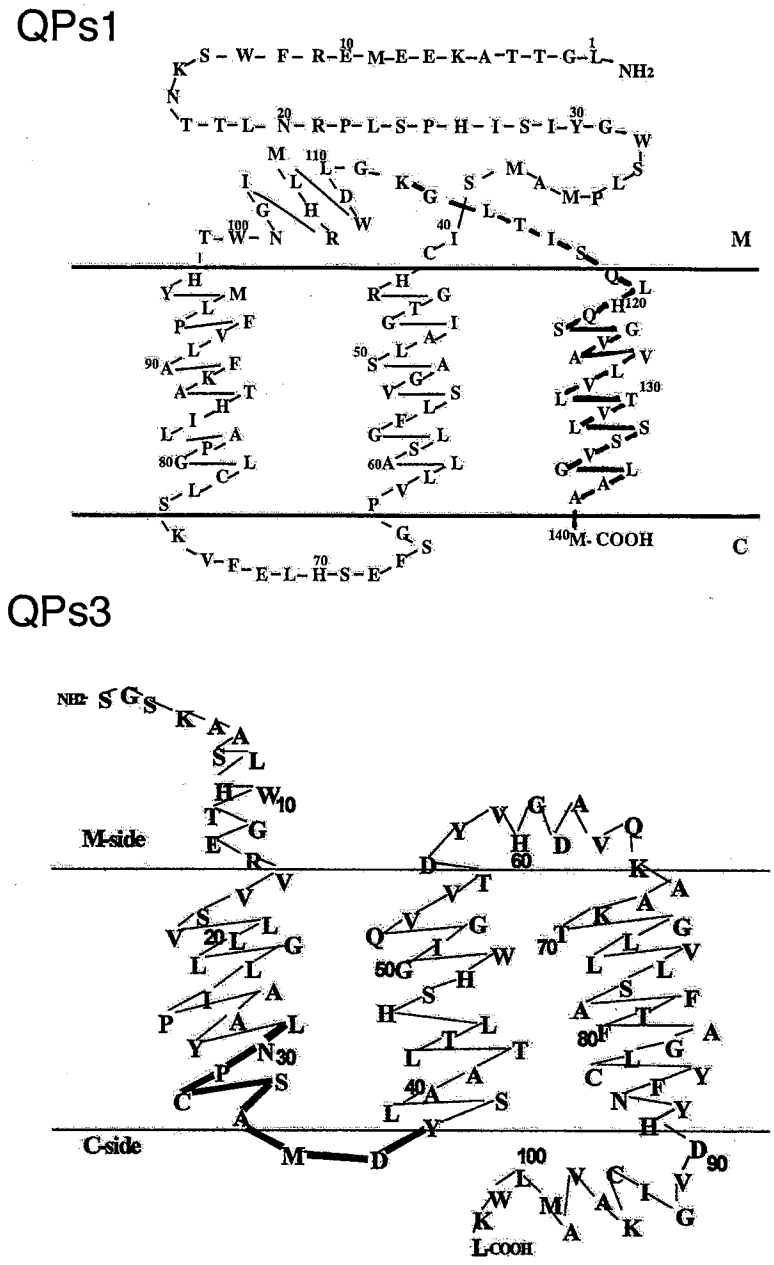


Fig. 2. Secondary structure of QPs1 and QPs3 from bovine heart mitochondrial SQR. Bold face regions are Q-binding domains identified by photoaffinity labeling.

not been identified.

It has been suggested that there are two Q-binding sites in SQR. A proximal Q-binding site is located close to the cytoplasm (matrix) of the membrane, in close contact with iron-sulfur cluster [3Fe-4S] (S3), as mentioned above. A distal Q-binding site is located close to the periplasm side of the membrane. Westenberg et al. (59) identified two clusters of amino acid residues in *E. coli* QFR that are responsible for reaction with quinone. One cluster is close to the cytoplasm side and the other is close to the periplasm side of the membrane.

The amino acid residues in putative Q-binding domain of QPs1 and QPs3 responsible for Q-binding have not yet been identified. The photoaffinity labeling technique does not permit us to identify specifically the amino acid residue(s) directly involved in Q-binding because of the rather long life and somewhat nonspecific nature of the activated nitrene radical. To use a molecular genetic approach to identify amino acid residues involved in Q-binding of bovine heart mitochondrial succinate-Q reductase, one needs to carry out site-directed mutagenesis of the QPs1 or QPs3 gene, expression and isolation of recombinant mutant protein, and reconstitution of recombinant mutant QPs1 or QPs3 with a QPs1 or QPs3 deficient succinate-Q reductase to form succinate-Q reductase. Although bovine heart mitochondrial QPs1 and QPs3 have been cloned and over-expressed in *E. coli* as GST fusion proteins (10,11), the functional activity of recombinant protein cannot be assessed because of the unavailability of reconstitutively active, QPs1 or QPs3-depleted succinate-Q reductase.

On the other hand, the *E. coli* SQR has become an attractive model system to study the Q:protein interactions. It is structurally and functionally similar to bovine heart mitochondrial SQR. The genes of the four subunits of *E. coli* SQR are organized in *sdh* operon, which has been cloned and sequenced (7). The enzyme is purified through a simple procedure from a strain containing overexpressed SQR (42). The purified enzyme has very low endogenous quinone content (<5%) comparing to its mitochondrial

counterpart SQR (~50%), which is a key feature for the study of Q-derivative to protein interactions. These features make *E. coli* SQR an ideal system to solve the problems we encountered in the bovine system.

In this study, the *E. coli* succinate-Q reductase has been functionally disassembled into two reconstitutively active fractions: the succinate dehydrogenase and the membrane-anchoring fraction (SdhC-SdhD) (36). The role of the membrane-anchoring fractions involved in Q-binding, as well as the role of cytochrome b_{556} in the enzyme become evident from the reconstitution studies. Isolated succinate dehydrogenase is soluble and can catalyze electron transfer from succinate to artificial acceptors, but not its physiological acceptor. Addition of the membrane anchoring fraction to isolated succinate dehydrogenase forms membrane-bound succinate-Q reductase which catalyzes electron transfer from succinate to Q (36), indicating that SdhC-SdhD provide membrane docking for succinate dehydrogenase and Q-binding in succinate-Q reductase.

Another objective of this research is to elucidate the detail of the interaction of quinone with SQR. We studied the interaction through the identification of the Q-binding site in *E. coli* SQR by photoaffinity labeling and site-directed mutagenesis. In design of mutations for site-directed mutagenesis studies, we followed the patterns of Q:protein interaction in bacterial photosynthetic reaction centers, which function as a light-driven ubiquinone reductase. The detailed three-dimensional structures of bacterial reaction centers are available (60,61). Bacterial photosynthetic reaction centers from *Rhodopseudomonas viridis* and *Rhodobacter sphaeroides* contain three essential subunits, L, M, and H. The high-resolution X-ray structures reveal two Q-binding sites, a tight binding Q_A site and an exchangeable Q_B site. Q_A mediates one electron transfer at a time, while Q_B associate with protons and exchange with Q pool after receiving two consecutive electrons from Q_A. In both QA and QB site, the carbonyl groups of quinone form hydrogen bonds with amino acid residues surrounding the Q-binding pocket. The detail of the interaction will be discussed in the corresponding chapters.

Among other quinone-binding proteins, the Q-binding domains in bovine bc_1 complex are identified by photoaffinity labeling (62). Bacterial bc_1 complexes, such as in *R. sphearoides*, are studied by site-directed mutagenesis coupled with specific Q-binding site inhibitors (63). The exact amino acid residues involved in Q-binding still await the improvement of the X-ray structure of bovine bc_1 complex.

CHAPTER II

RESOLUTION AND RECONSTITUTION OF SUCCINATE-UBIQUINONE REDUCTASE FROM *ESCHERICHIA COLI*

SUMMARY

Isolated *E. coli* succinate-ubiquinone reductase is resolved into a reconstitutively active, two-subunit succinate dehydrogenase and a two-subunit membrane anchoring protein fraction (the SdhC-SdhD fraction) by alkaline (pH 10.2) treatment of the reductase in the presence of 1 M urea, followed by DEAE-Sepharose CL-6B column chromatography under anaerobic conditions. Isolated succinate dehydrogenase and the SdhC-SdhD fraction alone show no succinate-ubiquinone reductase activity. However, when a given amount of the SdhC-SdhD fraction is mixed with varying amounts of succinate dehydrogenase, or vice versa, succinate-ubiquinone reductase activity increases as the amount of succinate dehydrogenase or the SdhC-SdhD fraction added increases. Maximum reconstitution is obtained when the weight ratio of succinate dehydrogenase to the SdhC-SdhD fraction reaches 5.26. This ratio is slightly higher than the calculated value of 3.37, obtained by assuming one mole of succinate dehydrogenase reacts with one mole of SdhC and SdhD. The isolated SdhC-SdhD fraction contains 35 nmoles cytochrome b_{556} per mg protein. Unlike mitochondrial cytochrome b_{560}, the cytochrome b_{556} is reducible by succinate in the isolated and complex forms. Furthermore, cytochrome b_{556} in the isolated SdhC-SdhD fraction has absorption properties, carbon monoxide reactivity, and EPR characteristics

similar to those of cytochrome b_{556} in intact succinate-ubiquinone reductase, indicating that its heme environments are not affected by the presence of succinate dehydrogenase. However, the redox potential of cytochrome b_{556} in the SdhC-SdhD fraction (22 mV) increases slightly when complexed with succinate dehydrogenase (34 mV). No hybrid succinate-ubiquinone reductase is formed from mitochondrial QPs and *E. coli* succinate dehydrogenase or vice versa. However, the cytochrome b_{556} in *E. coli* SdhC-SdhD fraction is reducible by succinate in the presence of mitochondrial succinate dehydrogenase and the rate of cytochrome b_{556} reduction correlates with the reconstitutive activity of the mitochondrial succinate dehydrogenase.

INTRODUCTION

The *Escherichia coli* succinate-Q reductase catalyzes the succinate-dependent reduction of ubiquinone during aerobic respiration (3). The purified complex contains four protein subunits and has five redox prosthetic groups: one covalent bound FAD, three iron-sulfur clusters, and one protoheme IX, b_{556}. The larger two subunits are succinate dehydrogenase. The two smaller subunits are membrane-anchoring proteins which house cytochrome b_{556}. The ligand for cytochrome b_{556} is a bis-histidine (47-50). Both membrane-anchoring subunits are thought to be required for b_{556} heme ligation, are essential for attachment of soluble succinate dehydrogenase to the inner surface of the cytoplasmic membrane, and are required for the reduction of Q. The membrane-anchoring protein fraction is named QPs in the mitochondrial system (53). Although intensive study of mitochondrial succinate-Q reductase has generated a wealth of information (3,15,39), the electron transfer mechanisms in this region of the chain is not understood.

Although *E. coli* succinate-ubiquinone reductase is structurally and functionally similar to the mitochondrial enzyme, cytochrome b_{556} in the *E. coli* enzyme is fully reducible by succinate (42) while that in mitochondria is not (45), indicating that the role of

cytochrome *b* may differ in these two enzyme systems. In *E. coli*, cytochrome b_{556} may be involved in electron transfer, while in mitochondria cytochrome b_{560} plays a structural role. The latter was established by the restoration of the absorption properties, redox potential, and EPR characteristics of cytochrome b_{560} during formation of a TTFA-sensitive succinate-Q reductase from isolated QPs and succinate dehydrogenase (45).

The fact that the *E. coli sdh* operon has been cloned and sequenced, and the enzyme has been purified, makes this an attractive model system for structure-function studies of mitochondrial succinate-Q reductase. However, the difference in succinate reducibility of cytochrome *b* in these two reductases, together with the fact that *E. coli* SdhD bears no sequence similarity with QPs3 of beef mitochondria raises the possibility that interactions between succinate dehydrogenase and membrane anchoring subunits in these two systems are different. We have developed a method to resolve *E. coli* succinate-Q reductase into reconstitutively active succinate dehydrogenase and membrane-anchoring protein fraction (the SdhC-SdhD fraction). This, together with the availability of reconstitutively active SDH and QPs from beef heart mitochondria, enables us to compare the mode of interaction between SDH and membrane anchoring subunits in these two systems.

Herein we report the preparation of reconstitutively active succinate dehydrogenase and the SdhC-SdhD fraction from *E. coli* succinate-ubiquinone reductase. The physical properties and reconstitutive activity of purified succinate dehydrogenase and the SdhC-SdhD fraction from *E. coli* were measured and compared to their mitochondrial counterparts. Formation of hybrid succinate-ubiquinone reductase was tested with mitochondrial succinate dehydrogenase and *E. coli* SdhC-SdhD fraction and vice versa.

EXPERIMENTAL PROCEDURES

Materials: Thenoyltrifluoroacetone (TTFA), dichlorophenol-indophenol (DCPIP), and polyoxyethylene-9-lauryl ether (E9C12), fumaric acid, and succinic acid were obtained

from Sigma. DEAE-Sepharose CL-6B was from Pharmacia LKB Biotechnology Inc. Oxalic acid was from Aldrich. Other chemicals were of the highest purity commercially available.

2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone (Q_2) was synthesized in our laboratory as previously reported (64). Calcium phosphate was prepared according to Jenner (65) and mixed at a 3:1 ratio with cellulose powder prior to use in column chromatography. Bovine heart mitochondrial succinate-ubiquinone reductase (66), succinate dehydrogenase (67), and QPs (45) were prepared by the reported methods. *E. coli* K12 strain NM256/pGS133 (7), which has plasmid containing a 4.5-kb *Bam*HI fragment (*sdh*CDAB) inserted into a low copy number vector pLC339, was a gift from Dr. R. B. Gennis of the University of Illinois. Growth of *E. coli* NM256/pGS133 was carried out according to Koland *et al* (68). Cells were harvested, when OD_{660} reached 1.5-1.7, by centrifugation at 3,000 x g for 10 min and frozen at -80 °C. Membrane was prepared from frozen cell pastes essentially according to the method of Kita *et al* (42) and frozen at -80 °C until use.

Isolation and Purification of Succinate-Ubiquinone Reductase from the Membrane Preparation of *E. coli*/NM256/pGS133-- Twenty-eight ml of frozen membrane preparation, 10 mg/ml, in 10 mM Tris-Cl, pH 7.5, containing 10% sucrose and 3 mM EDTA were thawed and added with freshly prepared E9C12 (25% stock solution in H_2O) to a final concentration of 4%. The mixture was incubated at 0 °C with constant stirring for one hour, then 24 ml of 50 mM K/K phosphate buffer, pH 7.5, was added, and the mixture was centrifuged at 41,000 xg for 1 hr. The supernatant obtained was applied to a DEAE-Sepharose CL-6B column (3.5 x 5 cm) equilibrated with 20 mM Tris-HCl, pH 7.5, containing 1% E9C12 (w/v), and 1 mM phenylmethylsulfonyl fluoride (PMSF). The column was washed in sequence with: 100 mL of 20 mM Tris-HCl, pH 7.5, containing 1% E9C12 (w/v) and 1 mM PMSF; 100 mL of the same buffer containing 0.1 M NaCl; and a linear gradient formed from 500 ml of the same buffer containing 0.1 M NaCl and 500 ml

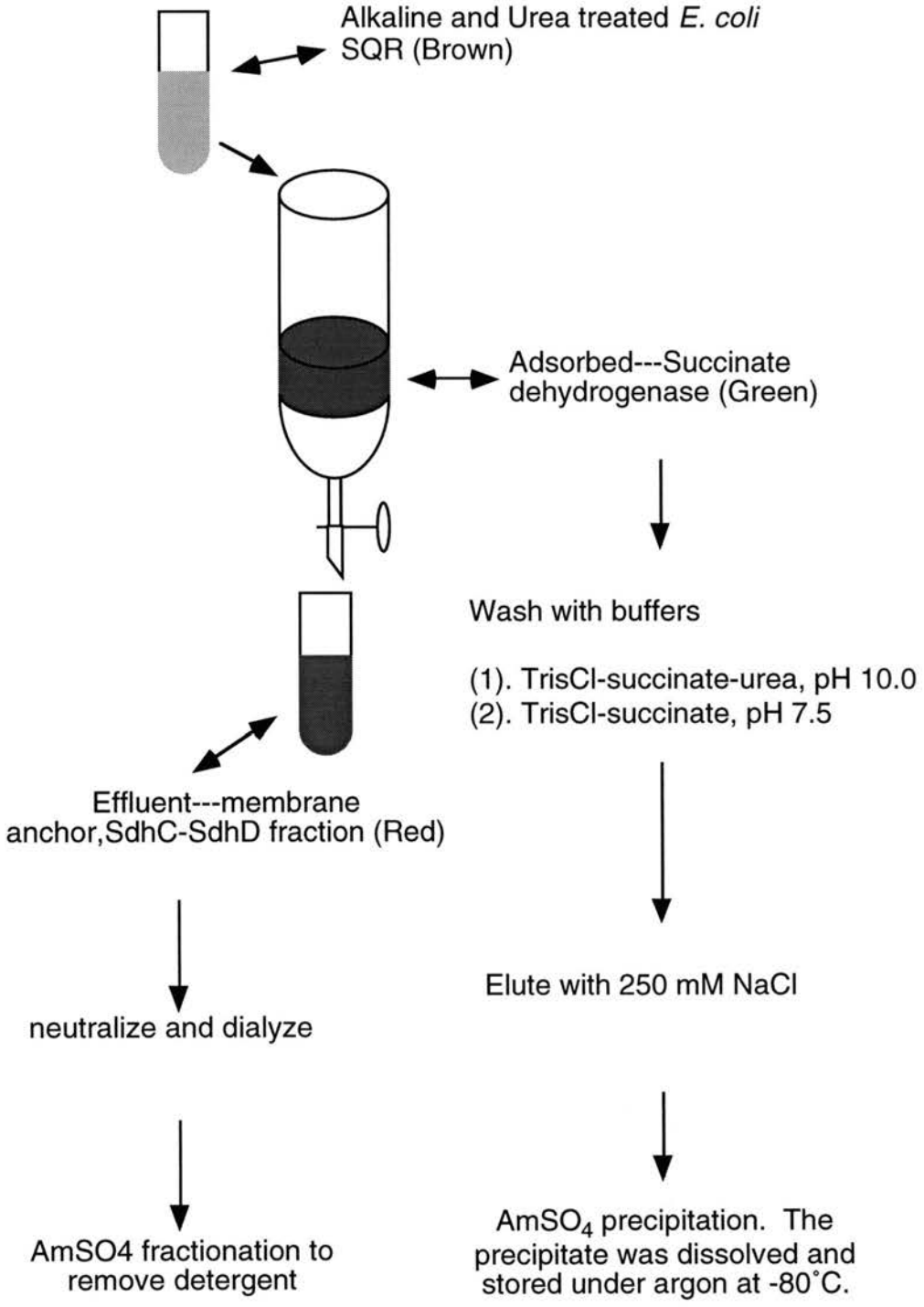
of the same buffer containing 0.25 M NaCl. The fractions containing succinate-ubiquinone reductase were collected, combined, and succinate added to a final concentration of 10 mM. The reductase was concentrated by precipitation with 35% ammonium sulfate saturation. The floating precipitate obtained was dissolved in 50 mM Tris-succinate buffer, pH 7.4, to a protein concentration of 10 mg/ml and used for preparation of succinate dehydrogenase and the SdhC-SdhD fraction.

Preparation of Succinate Dehydrogenase and the SdhC-SdhD fraction from *E. coli* Succinate-Ubiquinone Reductase-- Eight-ml of the isolated succinate-ubiquinone reductase, 10 mg/ml, in 50 mM Tris-succinate buffer, pH 7.4, was dialyzed against 800 ml of 30 mM Tris-Cl buffer, pH 7.5, containing 10 mM sodium succinate, for 4 hrs with one change of buffer. The dialyzed preparation was adjusted to a protein concentration of 6 mg/ml with 30 mM Tris-Cl buffer, pH 7.5, containing 10 mM sodium succinate, and flushed with argon for 30 minutes with stirring. All subsequent steps (Scheme VI) were carried out on ice or in the cold room (4 °C) under argon atmospheres. All solutions used were bubbled with argon gas for at least 10 min before use. Urea (8 M) solution was slowly added to the enzyme solution to a final concentration of 1 M. The mixture was then brought to pH 10.2 by adding 1 N NaOH dropwise. After incubation for 10 minutes the sample was loaded onto a DEAE-Sepharose CL-6B column (1.6 x 3 cm) equilibrated with 30 mM Tris-Cl, pH 10.0, containing 0.5 M urea and 10 mM sodium succinate. Succinate dehydrogenase was adsorbed to the column, while the SdhC-SdhD fraction were recovered in the effluent.

The column effluent was immediately adjusted to pH 7.0 with 1M Tris-Cl, pH 6.5, and dialyzed against 30 mM Tris-Cl, pH 7.5. The dialyzed *E. coli* SdhC-SdhD fraction was subjected to ammonium sulfate fractionation. The purified SdhC-SdhD fraction was recovered as a floating precipitate in the 10% and 35% ammonium sulfate fraction and redissolved in 30 mM Tris-Cl, pH 7.5.

The column containing adsorbed succinate dehydrogenase was washed with 2

Scheme VI. Preparation of succinate dehydrogenase and the SdhC-SdhD fraction from *E. coli* succinate-ubiquinone reductase



column volumes of 30 mM Tris-Cl, pH 10.0, containing 0.5 M Urea and 10 mM sodium succinate, followed by 30 mM Tris-Cl, pH 7.5, containing 10 mM sodium succinate until the pH of the eluate was neutral. This succinate dehydrogenase was eluted from the column with 30 mM Tris-Cl, pH 7.5, containing 250 mM NaCl and 10 mM sodium succinate. The succinate dehydrogenase fractions were combined and treated with ammonium sulfate to 50% saturation. The precipitate recovered was dissolved in a small volume of 50 mM Na/K phosphate buffer, pH 7.8 containing 20 mM succinate. This solution was divided into 100-µl portions and stored under argon at -80°C until use.

Succinate-ubiquinone reductase activity was assayed as previously reported (66). About 0.5 µg of enzyme complex was added to an assay mixture (1 ml) containing 50 mM Na/K phosphate, pH 7.0, 50 µM DCPIP, 20 mM succinate, 1 mM EDTA, 0.01% Triton X-100 and 25 µM of Q_2. Activity was determined by measuring the reduction of DCPIP (the decrease in absorbance at 600 nm). A millimolar extinction coefficient of 21 was used to calculate activity.

Absorption spectra were recorded with a Shimadzu spectrophotometer. The content of cytochrome b_{556} was determined from the reduced-minus-oxidized spectrum using the extinction coefficient of 22.8 cm^{-1} mM^{-1} for the wavelength pair 558-575 nm (42). Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Schägger *et al* (69) in which the 10% glycerol in the separating gel is replaced by 8 M urea (14).

Potentiometric titrations of cytochrome b_{556} in the isolated SdhC-SdhD fraction, and in intact and reconstituted succinate-ubiquinone reductases, were carried out anaerobically using the ferri-ferro-oxalate system, at pH 7.0 (midpoint redox potential = 0 mV), as described previously (70). EPR measurements were performed using a Bruker ER 200D spectrometer at liquid helium temperature. The instrument settings are given in the relevant figure legends.

RESULTS AND DISCUSSION

Isolation and Properties of *E. coli* Succinate-Ubiquinone Reductase-- Although a one-step purification procedure has been reported (42) for isolation of succinate-ubiquinone reductase from an *E. coli* strain containing the *sdh* operon on a pGS133 plasmid, the detergent, Lubrol PX, used in this method, is no longer commercially available. In order for this simple purification procedure to be useful, a substituting detergent for Lubrol PX must be found. Of 10 detergents tested, polyoxyethylene-9-lauryl ether (E9C12) was found to have the same effectiveness as Lubrol PX in solubilizing succinate-ubiquinone reductase from the membrane. Also, it is suitable for the subsequent DEAE-Sepharose CL-6B column chromatography. Succinate-ubiquinone reductase isolated with E9C12 has activity and purity comparable to that obtained with Lubrol PX. The isolated reductase catalyzes electron transfer from succinate to ubiquinone with a specific activity of 16 μmol succinate oxidized per min per mg protein (3.1 μmol succinate oxidized per min per nmol cytochrome b_{556}) at 23 °C. Activity was assayed by measuring the ability of the reductase to catalyze ubiquinone-mediated electron transfer from succinate to DCPIP. A maximum of 60% of the succinate-ubiquinone reductase activity detected in isolated succinate-Q reductase or in the membrane preparation is sensitive to TTFA (3 mM). This differs from that observed for the bovine heart mitochondrial succinate-ubiquinone reductase in which about 98% of the activity detected is sensitive to TTFA.

When the succinate-ubiquinone reductase obtained with E9C12 was subjected to SDS-PAGE using the system of Schägger *et al* (69), three major protein bands, with apparent molecular masses of 64, 28, and 14 kDa, and a very faint band with an apparent molecular mass of 13 kDa, were observed (see Fig. 3). This SDS-PAGE pattern is similar to that reported for the reductase prepared using Lubrol (42). These four protein bands are assumed to be SdhA (64,000), SdhB (28,000), SdhC (14,000), and SdhD (12,000), respectively (42). However, judging from Coomassie blue color intensities of the four

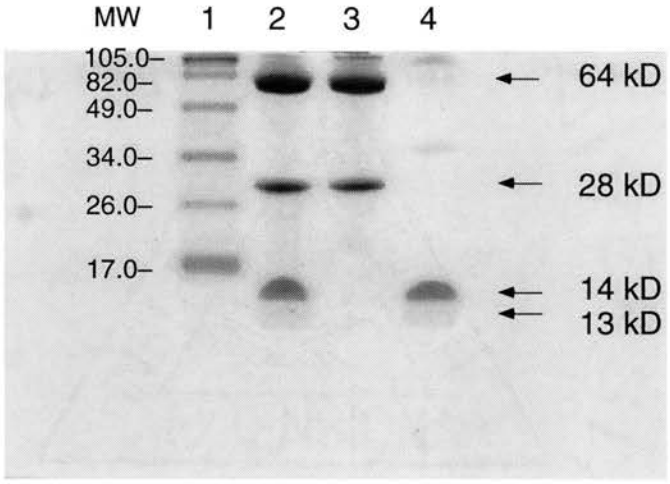


Fig. 3. SDS-PAGE of isolated succinate-ubiquinone reductase, succinate dehydrogenase, and the membrane-anchoring protein fraction. Lane 1, the molecular weight standard; lane 2, purified succinate-ubiquinone reductase; lane 3, purified succinate dehydrogenase; and lane 4, purified membrane-anchoring fraction.

protein bands, band IV is in substoichiometry to bands I and II of succinate dehydrogenase, while band III is in molar excess to bands I and II. The possibility that band IV is a contaminant protein and band III contains SdhC-SdhD has been ruled out because when the proteins in bands III and IV were eluted from the gel slices and subjected to the partial N-terminal amino acid sequence analysis, the sequences correspond to those of SdhC and SdhD, respectively. Thus, the band IV protein shown in SDS-PAGE is indeed SdhD. When the purified reductase is subjected to the partial-N-terminal amino acid sequence analysis, the percent recoveries of N-terminal amino acid residues of these four subunits are similar, indicating that the molar ratio of these four subunits in the purified reductase is 1:1:1:1 and the low color intensity of SdhD results from its low binding affinity with Coomassie blue dye because SdhD has low content of positively charged amino acid residues (5 out of 115 residues).

The isolated *E. coli* succinate-ubiquinone reductase contains 0.05 nmol Q per nmol cytochrome b_{556}, indicating that the isolated reductase is deficient in ubiquinone. Ubiquinone deficiency in succinate-ubiquinone reductase preparations is also evident from the activity increase, in the Q-stimulated DCPIP reduction by succinate, in the presence of excess Q_2. With 25 μM Q_2 in the assay mixture, *E. coli* succinate-ubiquinone reductase shows a more than 15-fold increase in DCPIP reduction (from 0.9 to 16 μmol succinate oxidized per min per mg protein), while the mitochondrial enzyme shows only a 2 to 2.5-fold increase (from 6.4 to 14 μmol succinate oxidized per min per mg protein), indicating a Q deficiency in the *E. coli* enzyme preparation of about 94% and in mitochondrial enzyme of about 40-50%. Since studying Q:protein interactions using synthetic ubiquinone derivatives requires prior removal of Q from the enzyme complex, the *E. coli* succinate-ubiquinone reductase preparation is superior to the mitochondrial enzyme preparation.

Resolution of Succinate-Ubiquinone Reductase into Succinate Dehydrogenase and the Membrane-anchoring Protein Fraction (the SdhC-SdhD fraction) -- Succinate-ubiquinone reductase is resolved into succinate dehydrogenase and the SdhC-SdhD fraction

by a procedure involving alkaline (pH 10.2) treatment, in the presence of 1 M urea, followed by DEAE-Sepharose CL-6B column chromatography. All steps are carried out under anaerobic conditions (argon atmosphere) and all reagents are bubbled with argon gas for at least 10 min before use. The alkaline pH, together with urea, splits succinate dehydrogenase from the SdhC-SdhD fraction and these two fractions are separated on a DEAE-Sepharose CL-6B column. The dark green colored succinate dehydrogenase is tightly absorbed to the column, while the reddish colored SdhC-SdhD fraction is recovered in the effluent. The absorbed succinate dehydrogenase was eluted with 250 mM NaCl. The greenish colored material remaining on the column that could not be eluted, even with 1 M salt, was identified as denatured succinate dehydrogenase. The denaturation of succinate dehydrogenase does not occur in the column chromatographic step because when DEAE-Sepharose CL-6B gel is used in a batch-wise eluting manner, instead of in a column, denatured succinate dehydrogenase is also observed. Prolonging the column chromatographic step increases the amount of succinate dehydrogenase denatured. In general, for a 1.5 hour-long column chromatographic step, the recovery of succinate dehydrogenase is about 60-70%, whereas the recovery of the SdhC-SdhD fraction is nearly 100%.

The pH of SdhC-SdhD containing effluent from the DEAE-Sepharose CL-6B column must be neutralized immediately. Urea in the effluent is removed by dialysis. Since dialysis does not remove E9C12, a relatively high concentration of detergent (2%) is present in the SdhC-SdhD preparation. High detergent concentrations destabilize cytochrome b_{556}, and make it very difficult to concentrate samples for use in experiments needing high protein concentrations, such as EPR. Ammonium sulfate fractionation of the dialyzed SdhC-SdhD fraction removes the excess E9C12 from the preparation as a thick white waxy floating precipitate formed between 5 and 20% ammonium sulfate saturation. The pure SdhC-SdhD fraction is recovered as a floating red precipitate formed between 20 to 35% ammonium sulfate saturation and is dissolved in a small volume of buffer to give a

protein concentration of 10-20 mg/ml. About 60% of the SdhC-SdhD in succinate-ubiquinone reductase is recovered in this final step. The SdhC-SdhD fraction thus obtained is ready for use in EPR experiments and can be stored at -80 °C for months without loss of reconstitutive activity .

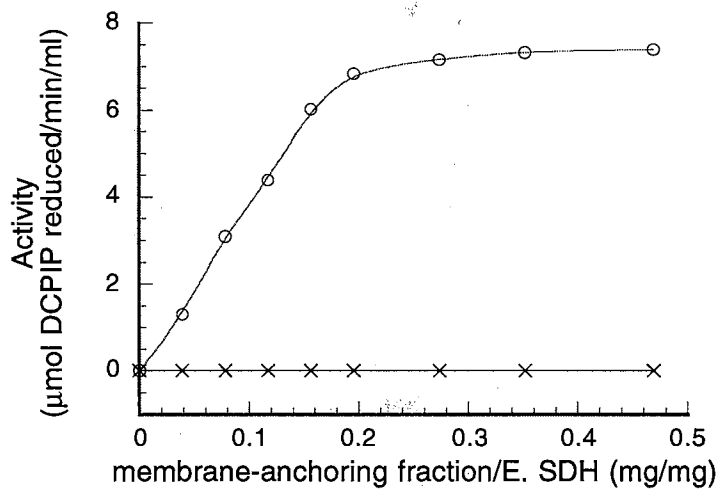
Purity and Reconstitutive Activity of *E. coli* succinate Dehydrogenase and Membrane-Anchoring Protein Fraction-- Isolated succinate dehydrogenase shows two protein bands on SDS-PAGE, with apparent molecular weights of 64 and 28 kDa (lane 3 of Fig. 3), corresponding to SdhA and SdhB in succinate-ubiquinone reductase. The isolated membrane-anchoring fraction shows two protein bands with apparent molecular masses of 14 and 13 kDa (lane 4 of Fig. 3), corresponding to SdhC and SdhD. The purity of isolated succinate dehydrogenase and membrane-anchoring protein fraction, judging from SDS-PAGE, is more than 90%.

Fig. 4 shows the reconstitution of succinate-ubiquinone reductase from succinate dehydrogenase and the SdhC-SdhD fraction. Isolated succinate dehydrogenase or the SdhC-SdhD fraction alone has no succinate-ubiquinone reductase activity. However, when a given amount of succinate dehydrogenase is mixed with varying amounts of the SdhC-SdhD fraction, the succinate-ubiquinone reductase activity increases as the amount of the SdhC-SdhD fraction used is increased (Fig. 4A). Maximal reconstitution is obtained when the weight ratio of succinate dehydrogenase to the SdhC-SdhD fraction reaches 5.26. The maximum reconstituted activity is 3.1 μmoles succinate oxidized per min per nmole cytochrome b_{556} at 23 °C. Addition of 3 mM TTFA in the assay mixture inhibits 50% of the reconstituted activity. Since the maximum reconstituted activity obtained and the degree of TTFA sensitivity are the same as those observed for intact succinate-ubiquinone reductase, the resolved succinate dehydrogenase and SdhC-SdhD fraction are reconstitutively active.

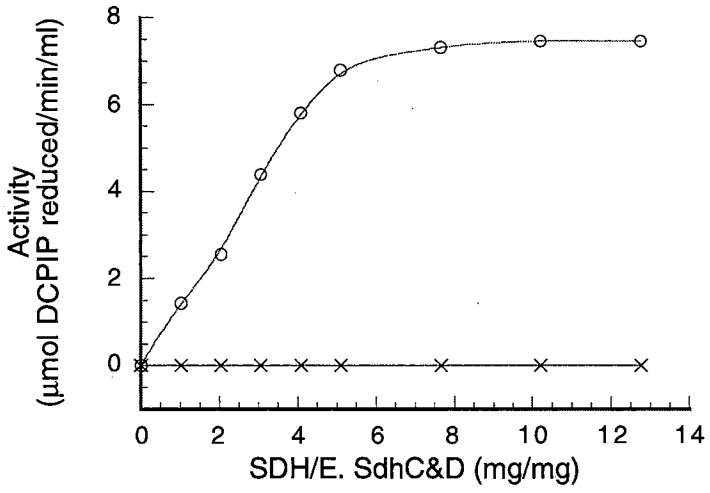
The weight ratio of 5.26 for maximum activity is also obtained when the reconstitution is performed with a given amount of the SdhC-SdhD fraction and varying

Fig. 4. Reconstitution of succinate-ubiquinone reductase by the membrane-anchoring protein fraction and succinate dehydrogenase. (A). Reconstitution of succinate-ubiquinone reductase made with a constant amount of *E. coli* succinate dehydrogenase and varying amounts of the membrane- anchoring protein fraction from *E. coli* (o) and from bovine heart mitochondria (x). 100-µl aliquots of 50 mM Na/K phosphate buffer, pH 7.8, containing indicated concentrations of the membrane-anchoring protein fraction were mixed with 10-µl aliquots of succinate dehydrogenase, 3.4 mg/ml, in 50 mM Na/K phosphate buffer, pH 7.8. The mixtures were incubated for 1 hr at 0 °C and succinate-ubiquinone reductase activity was assayed. (B). Reconstitution of succinate-ubiquinone reductase was made with a constant amount of *E. coli* membrane-anchoring protein fraction and varying amounts of succinate dehydrogenase from *E. coli* (o) and from bovine heart mitochondria (x). Ten-µl aliquots of *E. coli* membrane-anchoring protein fraction, 0.67 mg/ml, in 50 mM Na/K phosphate buffer, pH 7.8, were mixed with 100- µl of 50 mM Na/K phosphate buffer, pH 7.8, containing indicated concentrations of succinate dehydrogenase from *E. coli* (o) or bovine heart mitochondria (x). Succinate-ubiquinone reductase activity was measured after the mixtures were incubated at 0 °C for one hr.

A


Activity
(µmol DCPIP reduced/min/ml)
8
6
4
2
0
0
0.1
0.2
0.3
0.4
0.5
membrane-anchoring fraction/E. SDH (mg/mg)

B


Activity
(µmol DCPIP reduced/min/ml)
8
6
4
2
0
0
2
4
6
8
10
12
14
SDH/E. SdhC&D (mg/mg)

amounts of succinate dehydrogenase (Fig. 4B). If intact succinate-ubiquinone reductase contains 4 subunits, with molecular masses of 64,268, 26,637, 14,167, and 12,792, in equal molar ratio, the weight ratio between succinate dehydrogenase and the SdhC-SdhD fraction should be 3.37. That the experimental value is higher than the calculated value is probably due to slight denaturation of the soluble succinate dehydrogenase used, since it is known to be labile in the soluble form.

Absorption Spectra, Redox Potential, Carbon Monoxide Reactivity, and EPR Characteristics of Cytochrome b_{556}-- The isolated *E. coli* SdhC-SdhD fraction contains 35 nmoles cytochrome b_{556} per mg protein. The oxidized cytochrome shows broad absorption at the α-and β-regions with Soret absorption at 412 nm (see Fig. 4). When the sample is reduced with dithionite, an α-absorption at 558 nm, a broad β-absorption peak at between 526 and 528 nm, and the Soret absorption at 424 nm are observed (Fig. 5). These absorption characteristics are identical to those observed for cytochrome b_{556} in intact or reconstituted *E. coli* succinate-ubiquinone reductase, indicating that the absorption characteristics of cytochrome b_{556} are not affected by the presence of succinate dehydrogenase. This is in contrast to the bovine heart mitochondrial system in which the spectral characteristics of cytochrome b_{560} in isolated QPs differs from those of cytochrome b_{560} in the reductase (Fig. 6). The dithionite-reduced cytochrome b_{560} in isolated mitochondrial QPs has a symmetrical α-absorption peak at 560 nm whereas in succinate-ubiquinone reductase it exhibits an α-absorption maximum at 560.5 nm with a discernible shoulder at 553 nm. Addition of succinate dehydrogenase to QPs to form succinate-ubiquinone reductase regenerates the 553 nm shoulder peak, indicating that the heme environments of cytochrome b_{560} are affected by the presence of succinate dehydrogenase (Fig. 6).

Cytochrome b_{556} in isolated succinate-ubiquinone reductase is reducible by succinate. Cytochrome b_{556} in the isolated SdhC-SdhD fraction is also reducible by succinate in the presence of succinate dehydrogenase under anaerobic conditions (data not

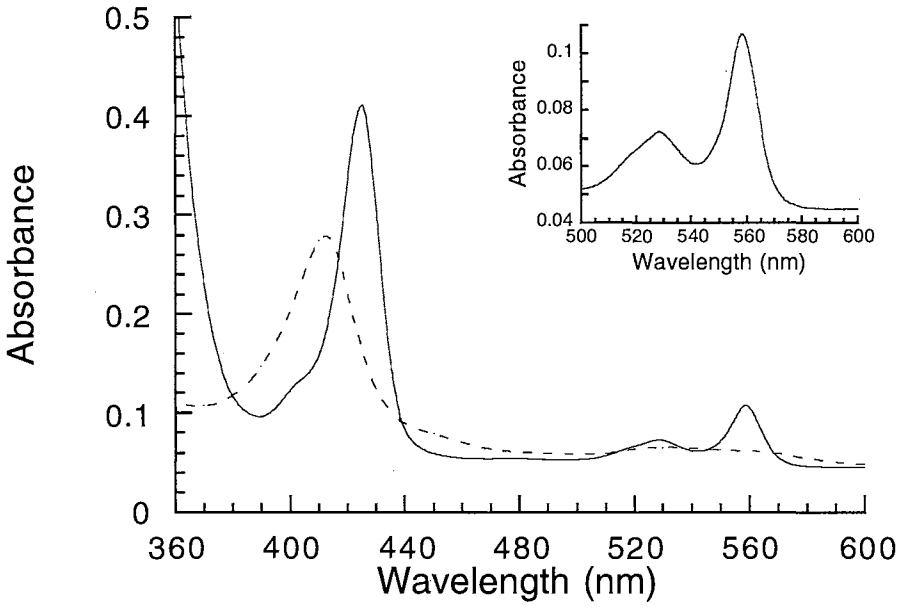


Fig. 5. Absorption spectra of isolated cytochrome b_{556}. The broken line (- -) represents the preparation as isolated, and the solid line (—) represents this preparation reduced by dithionite. Insert is the difference spectrum of reduced minus oxidized form at the α-and β-regions.

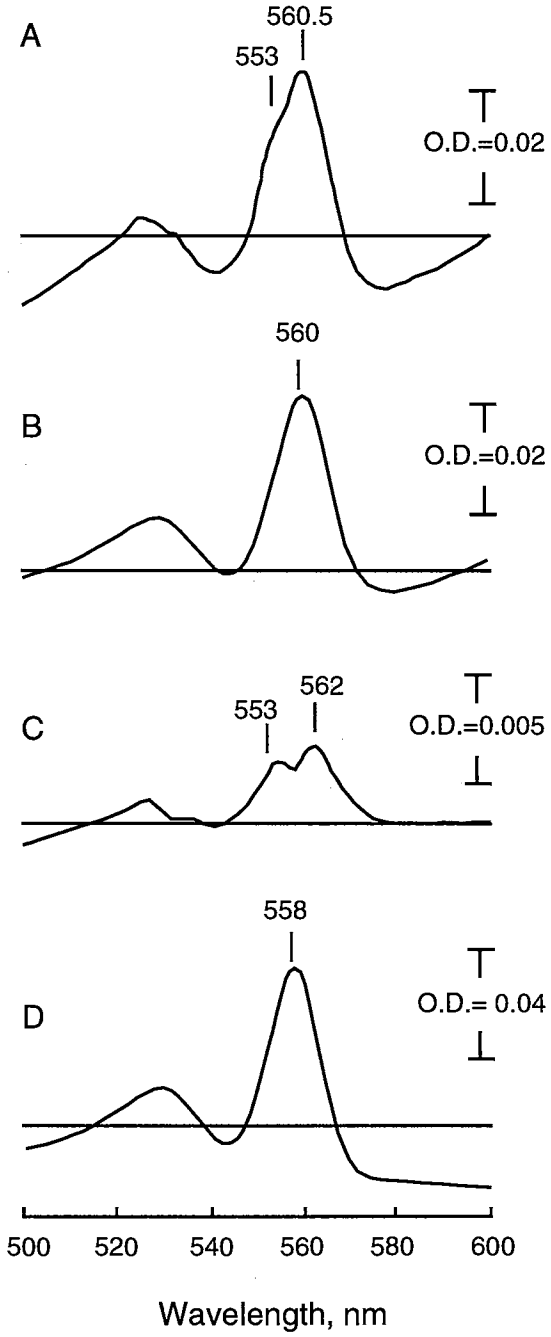


Fig. 6. Comparison of the difference spectra of cytochrome *b* of SQR from mitochondria and *E. coli* in different isolation states. A. bovine heart mitochondrial cytochrome b_{560} in intact SQR, B, in isolated QPs, C, in reconstituted SQR. D, *E. coli* cytochrome b_{556} in intact SQR, isolated SdhC-SdhD, and reconstituted SQR.

shown). The succinate reduced spectrum of cytochrome b_{556}, either in the isolated SdhC-SdhD fraction or in succinate-ubiquinone reductase, is the same as that obtained with dithionite. On the other hand, cytochrome b_{560} in intact mitochondrial succinate-ubiquinone reductase or isolated QPs is not reducible by succinate.

Reduced cytochrome b_{556} and cytochrome b_{560} in isolated succinate-ubiquinone reductases from *E. coli* and bovine heart mitochondria, respectively, do not react with carbon monoxide (Table I). Reduced cytochrome b_{556} in the isolated *E. coli* SdhC-SdhD fraction does not react with carbon monoxide, while cytochrome b_{560} in isolated mitochondrial QPs, in its reduced form, is completely reactive with carbon monoxide (Table I). Addition of mitochondrial succinate dehydrogenase to mitochondrial QPs converts carbon monoxide reactive cytochrome b_{560} in QPs into a carbon monoxide non-reactive form. As expected, after addition of *E. coli* succinate dehydrogenase to the *E. coli* SdhC-SdhD fraction, cytochrome b_{556} in the SdhC-SdhD fraction remains totally non-reactive toward carbon monoxide. These results suggest that the environments of the fifth and sixth ligands of mitochondrial cytochrome b_{560} differ from those of *E. coli* cytochrome b_{556}, even though the ligands for both cytochromes are bis-histidines (50, 49).

The midpoint potential of cytochrome b_{556} in the isolated *E. coli* SdhC-SdhD fraction is estimated to be +22 mV, about 12 mV lower than that of cytochrome b_{556} in intact (34 mV) succinate-ubiquinone reductases. Addition of succinate dehydrogenase to the SdhC-SdhD fraction increases the midpoint potential of cytochrome b_{556} to 33 mV with concurrent reconstitution of succinate-ubiquinone reductase. The redox potential of *E. coli* cytochrome b_{556} in the isolated SdhC-SdhD fraction is 166 mV higher than that of cytochrome b_{560} in isolated mitochondrial QPs (-144 mV); and the redox potential of cytochrome b_{556} in intact *E. coli* succinate-ubiquinone reductase is 219 mV higher than that of cytochrome b_{560} in intact mitochondrial succinate-ubiquinone (-185 mV) reductase. These values correlate with the fact that mitochondrial cytochrome b_{560} is not reducible by succinate in either the isolated or complex form, while *E. coli* cytochrome b_{556} is reducible

TABLE I. COMPARISON OF CO REACTIVITY OF REDUCED CYTOCHROME *b* FROM *E. COLI* SQR AND BOVINE HEART MITOCHONDRIAL SQR IN DIFFERENT ISOLATION STATES.

Preparations	Reduced cytochrome *b*	
	E. coli	Bovine heart mitochondria
In intact SQR	Not reactive*	Not reactive
In isolated membrane-anchoring fraction	Not reactive	Reactive
In reconstituted SQR	Not reactive	Not reactive

* CO reactivity is determined by flushing the enzyme solution with a stream of CO for 5 minutes. The spectrum of the enzyme solution is recorded for 500-600 nm and compared to the one before CO treatment.

by succinate in both forms. Like mitochondrial cytochrome b_{560}, the redox potential of *E. coli* cytochrome b_{556} is affected by the interaction of SdhC-SdhD with succinate dehydrogenase (Table II).

Fig. 7 compares EPR characteristics of cytochromes *b* in isolated membrane-anchoring fraction, intact and reconstituted succinate-ubiquinone reductases from *E. coli* and mitochondrial systems. Cytochrome b_{556} in the *E. coli* SdhC-SdhD fraction shows an EPR signal at g=3.65 (Fig. 7A). This signal is identical to that of cytochrome b_{556} in intact and reconstituted *E. coli* succinate-ubiquinone reductases, indicating that removal of succinate dehydrogenase did not affect the heme environments of cytochrome b_{556}. This is different from that observed for mitochondrial cytochrome b_{560}. Cytochrome b_{560} in intact succinate-ubiquinone reductase exhibits a EPR signal at g=3.46, while it shows two EPR signals at g=3.07 and 2.92 in isolated QPs (Fig. 7B). Addition of succinate dehydrogenase to QPs converts the g=3.07 EPR signal of cytochrome b_{560} in QPs to g=3.46, indicating that the heme environment of cytochrome b_{560} is affected by the interaction of QPs with succinate dehydrogenase.

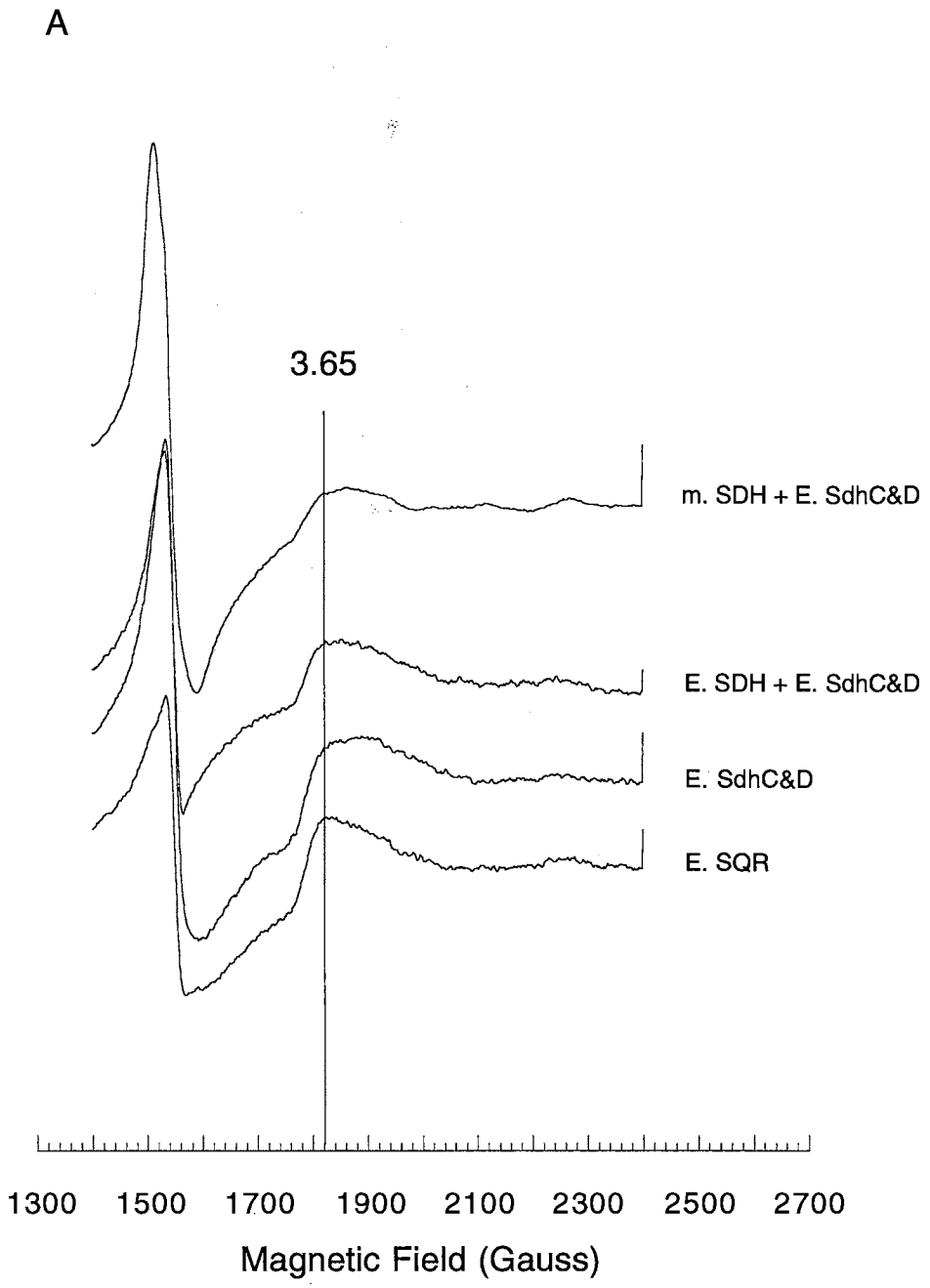
Formation of Hybrid Succinate-Ubiquinone Reductase by the *E. coli* Membrane-anchoring Fraction and Mitochondrial Succinate Dehydrogenase and Vice Versa-- The availability of isolated, reconstitutively active membrane-anchoring fraction and succinate dehydrogenase from the *E. coli* and bovine mitochondrial systems enables us to study the formation of a hybrid succinate-ubiquinone reductase from the *E. coli* membrane-anchoring protein fraction and mitochondrial succinate dehydrogenase and vice versa. When a given amount of *E. coli* succinate dehydrogenase is mixed with increasing amounts of mitochondrial QPs, no succinate-ubiquinone reductase activity is detected (see Fig. 4A, cross symbols). Failure to reconstitute is also observed when the *E. coli* SdhC-SdhD fraction is mixed with increasing amounts of mitochondrial succinate dehydrogenase (see Fig. 4B, cross symbols).

Since no hybrid succinate-ubiquinone reductases are formed, it is of interest to see

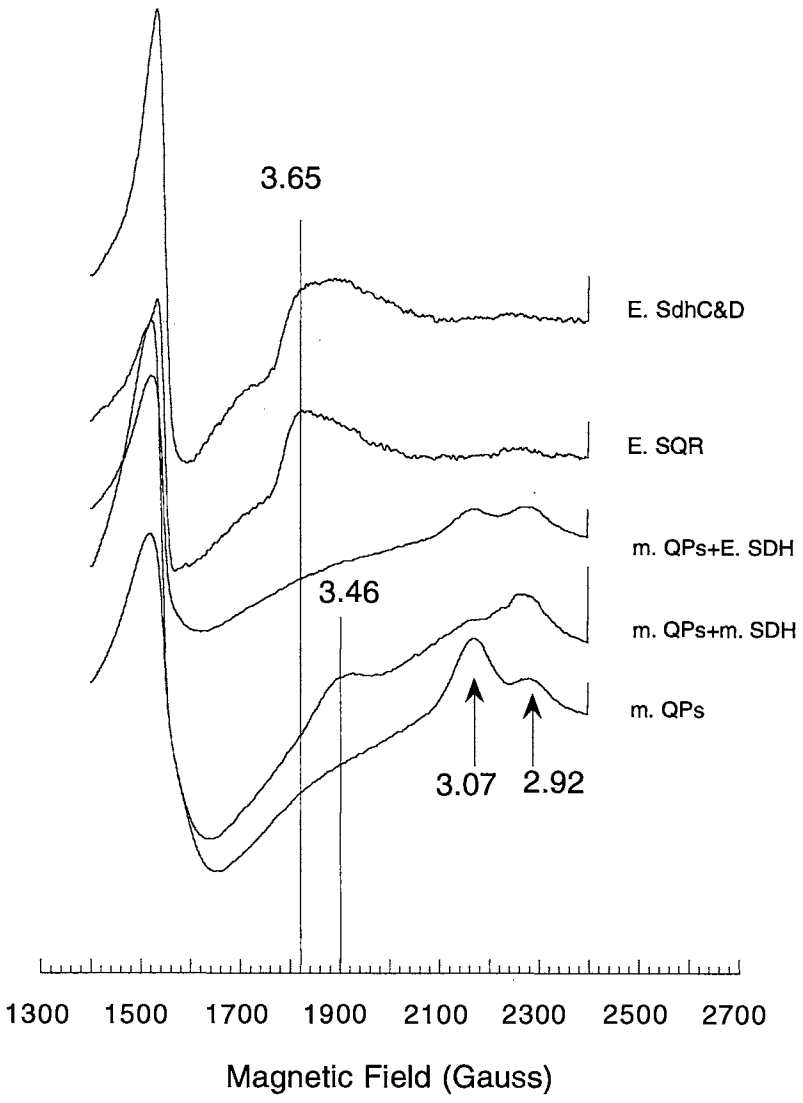
TABLE II. COMPARISON OF MIDPOINT POTENTIALS OF CYTOCHROME *b* FROM *E. COLI* SQR AND BOVINE HEART MITOCHONDRIAL SQR IN DIFFERENT ISOLATION STATES.

Preparations	Midpoint potential of cytochrome *b* (mV)	
	E. coli	Bovine heart mitochondria
In intact SQR	+34	-185
In isolated membrane-anchoring fraction	+22	-144
In reconstituted SQR	+33	-168

Fig. 7. Effect of succinate dehydrogenase on EPR spectra of cytochrome b_{560} in QPs from bovine heart mitochondria and of cytochrome b_{556} in the SdhC-SdhD fraction from *E. coli.* (A). EPR spectra of cytochrome b_{556} in *E. coli* SdhC-SdhD fraction in the presence and absence of succinate dehydrogenases from *E. coli* and bovine heart mitochondria. 0.2-ml aliquots of the *E. coli* SdhC-SdhD fraction, 800 μM cytochrome b_{556}, in 50 mM Na/K phosphate buffer, pH 7.4, were mixed with 0.2 ml of 50 mM Na/K phosphate buffer, pH 7.8, containing none, 27 mg of succinate dehydrogenase from bovine heart mitochondria, and 28 mg of succinate dehydrogenase from *E. coli*. The mixtures were incubated at 0 °C for one hour and assayed for succinate-ubiquinone reductase activity before being placed in the EPR tubes and frozen at -80 °C. (B). EPR spectra of cytochrome b_{560} in bovine heart mitochondrial QPs in the presence and absence of succinate dehydrogenases from *E. coli* and bovine heart mitochondria. 0.2 ml aliquots of QPs were added 0.2 ml of 50 mM Na/K phosphate buffer, pH 7.4, containing none, 25 mg of *E. coli* succinate dehydrogenase, and 27 mg of bovine heart mitochondrial succinate dehydrogenase. The mixtures were incubated at 0 °C for one hour and assayed for succinate-ubiquinone reductase activity before being placed in EPR tubes and frozen at -80 °C. The EPR instrument settings were: modulation frequency, 100 KHz; modulation amplitude, 20G; time constant, 0.5s; microwave frequency, 9.42 GHz; microwave power, 20 milliwatts; scan rate, 200 s; and temperature, 11 K.


A
3.65
m. SDH + E. SdhC&D
E. SDH + E. SdhC&D
E. SdhC&D
E. SQR
1300
1500
1700
1900
2100
2300
2500
2700
Magnetic Field (Gauss)

B


3.65
E. SdhC&D
E. SQR
m. QPs+E. SDH
3.46
m. QPs+m. SDH
m. QPs
3.07
2.92
1300
1500
1700
1900
2100
2300
2500
2700
Magnetic Field (Gauss)

whether or not this is due to a lack of interaction between succinate dehydrogenase and the membrane-anchoring protein fraction from these two systems. The interaction can be indicated by reduction of cytochrome b_{556} in the isolated *E. coli* SdhC-SdhD fraction in the presence of mitochondrial succinate dehydrogenase or a change of EPR characteristics of cytochrome b_{560} in isolated mitochondrial QPs upon the addition of *E. coli* succinate dehydrogenase. The first indication of interaction is based on the fact that cytochrome b_{556} in the isolated *E. coli* SdhC-SdhD fraction is reducible by succinate upon the addition of *E. coli* succinate dehydrogenase. The second indication of interaction is based on the observation that one of the EPR signals (g=3.07) of cytochrome b_{560} in isolated mitochondrial QPs converts to a g=3.46 signal upon the addition of mitochondrial succinate dehydrogenase.

Table III shows that the cytochrome b_{556} in *E. coli* SdhC-SdhD fraction is reducible by succinate in the presence of reconstitutively active mitochondrial or *E. coli* succinate dehydrogenase. The rate of reduction correlates with the reconstitutive activity of the added succinate dehydrogenase. When the isolated *E. coli* SdhC-SdhD fraction is mixed with mitochondrial succinate dehydrogenases having decreasing low K_m-ferricyanide reductase activity, the rate of cytochrome b_{556} reduction by succinate decreases correspondingly. No cytochrome b_{556} reduction is observed when a mitochondrial succinate dehydrogenase possessing no low-K_m ferricyanide reductase activity is added to the *E. coli* SdhC-SdhD fraction. Since the low-K_m ferricyanide reductase activity in soluble succinate dehydrogenase correlates with its reconstitutive activity, the rate of cytochrome b_{556} reduction by succinate in the *E. coli* SdhC-SdhD fraction correlates with the reconstitutive activity of succinate dehydrogenase. Succinate dehydrogenases with decreasing low-K_m ferricyanide reductase activity are obtained by exposing fully reconstitutively active soluble succinate dehydrogenase to air for increasing lengths of time. The rate of cytochrome b_{556} reduction by succinate is faster with mitochondrial succinate dehydrogenase than with the *E coli* enzyme, at the same protein concentration. This is in

TABLE III. REDUCTION OF CYTOCHROME b_{556} IN THE *E. COLI* SDHC-SDHD FRACTION BY VARIOUS PREPARATIONS

Preparation Added	Reduction of cyt. b_{556}	Reduction Rate
		μmol b_{556} /min/mg
E. coli SQR	+	0.27
Mito SQR	-	-
E. coli SDH	+	0.31
Mito SDH (Reconst.active)	+	0.40
Mito SDH (Reconst. inactive)	-	-

SQR, succinate-ubiquinone reductase; SDH, succinate dehydrogenase.

line with the fact that isolated succinate dehydrogenase from bovine heart mitochondria has a higher low-K_m ferricyanide activity than does succinate dehydrogenase from *E. coli*. As expected, no reduction of cytochrome b_{556} by succinate is observed when mitochondrial succinate-ubiquinone reductase or succinate-cytochrome *c* reductase is added to the *E. coli* SdhC-SdhD fraction because the succinate dehydrogenase in these reductase complexes contains no low-K_m ferricyanide reductase activity (or reconstitutive activity).

Since cytochrome b_{556} is not reducible by succinate in the presence of reconstitutively inactive succinate dehydrogenase or intact succinate-ubiquinone reductase from bovine heart mitochondria, the electron for the reduction of cytochrome b_{556} in *E. coli* SdhC-SdhD fraction must come from the low-K_m ferricyanide reductase site of succinate dehydrogenase. These results indicate that succinate dehydrogenase from bovine heart mitochondria can interact with the *E. coli* SdhC-SdhD fraction and reduce cytochrome b_{556} at the expense of succinate. However, this reduced cytochrome b_{556} is unable to reduce Q and thus no succinate-ubiquinone reductase is formed. The inability of reduced cytochrome b_{556} to reduce Q may indicate that cytochrome b_{556} in *E. coli* succinate-ubiquinone reductase is not the donor of the first electron for Q. Possibly, the electron acceptor for cytochrome b_{556} is a ubisemiquinone radical and the donor of the first electron for Q is *E. coli* succinate dehydrogenase. In the hybrid system, the mitochondrial succinate dehydrogenase is unable to provide the first electron to reduce Q to ubisemiquinone. Alternatively, the reduction of cytochrome b_{556} is not required for succinate-ubiquinone reductase activity in *E. coli* system.

Interaction between mitochondrial QPs and *E. coli* succinate dehydrogenase is suggested by the decrease in the signal height of the g=3.07 EPR signal of cytochrome b_{560} in QPs upon the addition of *E. coli* succinate dehydrogenase (see Fig. 7B). However, this interaction differs from that observed in the mitochondrial reconstituting system in which the g=3.07 EPR signal of cytochrome b_{560} in QPs is converted to a g= 3.46 signal. Since addition of *E. coli* succinate dehydrogenase to mitochondrial QPs causes a decrease

in the g=3.07 signal height of cytochrome b_{560} without producing the g=3.46 signal, no succinate-ubiquinone reductase activity is reconstituted. This suggests that *E. coli* succinate dehydrogenase interacts with mitochondrial QPs to some degree but not enough to form an active reductase.

CHAPTER III

IDENTIFICATION OF QUINONE-BINDING DOMAIN IN SUCCINATE-UBIQUINONE REDUCTASE FROM *ESCHERICHIA COLI* BY PHOTOAFFINITY LABELING

SUMMARY

When purified ubiquinone-depleted succinate-ubiquinone reductase from *E. coli* is photoaffinity labeled with 3-azido-2-methyl-5-methoxy[^{3}H]-6-geranyl-1,4-benzoquinone followed by SDS-PAGE, radioactivity is found in the SdhC subunit, indicating that this subunit is responsible for ubiquinone binding. An [^{3}H]azido-Q linked peptide, with retention time of 61.7 min, is obtained by high performance liquid chromatography of the protease K digest of [^{3}H]azido-Q labeled SdhC obtained from preparative SDS-PAGE on labeled reductase. The partial N-terminal amino acid sequence of this peptide is NH_2-T-I-R-F-P-I-T-A-I-A-S-I-L-H-R-V-S-, corresponding to residues 17-33. The ubiquinone-binding domain in the proposed structural model of SdhC, constructed based on the hydropathy plot of the deduced amino acid sequence of this protein, is located at the N-terminal end toward the transmembrane helix I.

INTRODUCTION

Escherichia coli succinate-Q reductase, which catalyzes electron transfer from

succinate to ubiquinone during aerobic respiration, has been purified and characterized (3). The *E. coli* succinate-Q reductase has been resolved into two reconstitutively active fractions: succinate dehydrogenase and the membrane-anchoring fraction (SdhC-SdhD) (36). Isolated succinate dehydrogenase is soluble and can catalyze electron transfer from succinate to artificial acceptors, such as phenazinemethosulfate, but not to its physiological acceptor, Q. Addition of the membrane anchoring fraction to isolated succinate dehydrogenase forms membrane-bound succinate-Q reductase which catalyzes electron transfer from succinate to Q (36), indicating that SdhC-SdhD provide membrane docking for succinate dehydrogenase and Q-binding in succinate-Q reductase. In fact, the membrane-anchoring protein fraction is named QPs (Q-binding protein in succinate-ubiquinone reductase) in the mitochondrial system (53). The involvement of membrane-anchoring fraction in the Q-binding of *E. coli* succinate-Q reductase is shown by detection of ubisemiquinone radicals in intact and reconstituted succinate-Q reductases in the presence of exogenous Q. No ubisemiquinone radical is detected with succinate dehydrogenase in the presence of Q.

The understanding of the protein:Q interaction and the mechanism of Q reduction in succinate-Q reductase requires knowledge of the Q-binding site. Photoaffinity labeling of beef heart mitochondrial succinate-Q reductase with [^{3}H]azido-Q derivative identified all three QPs subunits as putative Q-binding proteins in this reductase. The Q-binding domains in QPs1 is located at the loop connecting transmembrane helices II and III which extrudes from the surface of the M-side of the mitochondrial inner membrane (1). The Q-binding domain in QPs3 is located at the end of transmembrane helix I toward the C-side of the membrane (38). The Q-binding domain(s) in other succinate-Q reductases has not been identified.

The amino acid residues in the putative Q-binding domain of QPs1 and QPs3, responsible for Q-binding, have not been identified. Although bovine heart mitochondrial QPs1 and QPs3 have been cloned and over-expressed in *E. coli* as GST fusion proteins,

the functional activity of the recombinant protein awaits to be assessed. Since *E. coli* succinate-Q reductase is structurally and functionally similar to the mitochondrial enzyme, and is readily manipulated genetically, it provides us with an ideal system for the study of Q-binding site using photoaffinity labeling techniques in concert with site-directed mutagenesis. Herein, we report the identification of the Q-binding domain in *E. coli* succinate-Q reductase by photoaffinity labeling.

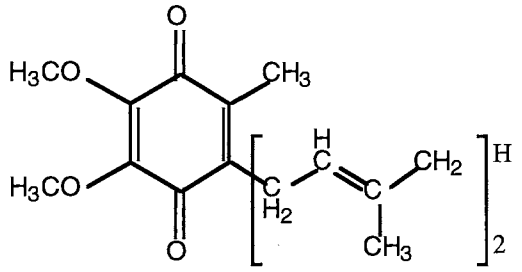
EXPERIMENTAL PROCEDURES

Materials: Dichlorophenol-indophenol (DCPIP), polyoxyethylene-9-lauryl ether (E9C12). DEAE-Sepharose CL-6B was from Pharmacia LKB Biotechnology Inc. Other chemicals were of the highest purity commercially available.

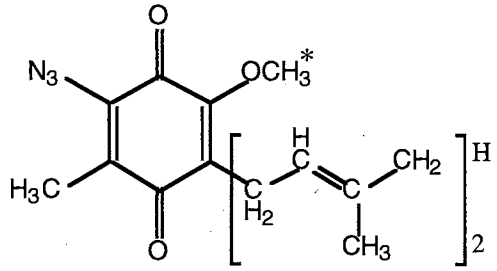
Ubiquinone derivatives (Scheme VII): 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone (Q_2), 3-azido-2-methyl-5-methoxy[^{3}H]-6-geranyl-1,4-benzoquinone ([^{3}H]azido-Q), 5-azido-2,3-dimethoxy[^{3}H]-6-geranyl-1,4-benzoquinone ([^{3}H]-5-azido-Q), were synthesized in our laboratory as previously reported (64).

Enzyme Preparations and Assays-- Membranes were prepared from EΔSQR complement and mutant strains as described previously (42). The succinate-Q reductase was purified from membranes by the method of Yang *et al.* (36). Succinate-Q reductase activity was assayed for its ability to catalyze Q-stimulated DCPIP reduction by succinate. The assay was performed at room temperature in a Shimadzu UV2101PC spectrophotometer. About 0.5 μg of enzyme was added to an assay mixture (1 ml) containing 50 mM sodium/potassium phosphate, pH 7.0, 50 μM DCPIP, 20 mM succinate, 1 mM EDTA, 0.01% Triton X-100 and 25 μM of Q_2. For determination of the apparent K_m for Q_2, various concentrations of Q_2 were used. The reduction of DCPIP was followed by measuring the absorbance decrease at 600 nm, using a millimolar extinction coefficient of 21 mM^{-1} cm^{-1}.

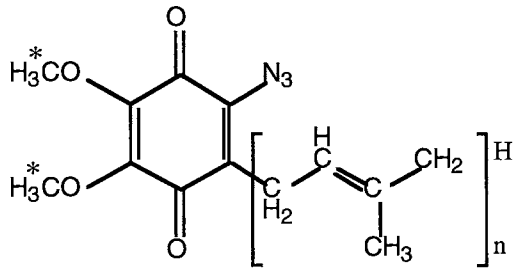
Scheme VII. Structures of ubiquinone-2 and azido-Q derivatives used in this study



Q_2



azido-Q



5-azido-Q

Isolation of [^{3}H]Azido-Q-labeled SdhC-- 8 ml of succinate-Q reductase, 2 mg/ml (10.6 μM cytochrome b_{556}), in 50 mM sodium/potassium phosphate buffer, pH 7.5, containing 0.1% E9C12 were incubated with 424 nmoles of [^{3}H]azido-Q (5 molar excess to cytochrome b_{556}) at 0 °C for 10 min in the dark. The [^{3}H]azido-Q was added in 100 μl ethanolic solution. The specific radioactivity of azido-Q used was 1.6 x 10^4 cpm/nmol in 95% ethanol and 6.6 x 10^3 cpm/nmol in 50 mM sodium/potassium phosphate buffer, pH 7.5, containing 0.1% E9C12 in the presence of succinate-Q reductase. This mixture was transferred to an illuminating apparatus made from two quartz glasses sandwiched by a Teflon ring which was immersed in ice in a Petri dish to maintain the temperature at 0 °C. The sample was illuminated with long wavelength UV light (Spectroline EN-14, 365 nm long wave length, 23 watts) for 7 min at a distance of 5 cm from the light source.

To determine the amount of [^{3}H]azido-Q incorporated into succinate-Q reductase, the illuminated [^{3}H]azido-Q treated samples were spotted onto a 3 M paper and developed with a mixture of chloroform and methanol (2:1, v/v) to remove non-protein bound [^{3}H]azido-Q. The paper at the origin was cut into small pieces and subjected to liquid scintillation counting.

The illuminated [^{3}H]azido-Q-treated succinate-Q reductase was precipitated by 50% ammonium sulfate saturation and centrifuged at 12,000 xg for 20 min. The precipitate was dissolved in 30 mM Tris-Cl buffer, pH 7.5, and dialyzed against double-distilled water, overnight, with one change of water. The dialyzed sample was subjected to organic solvent extraction as previously described (1), to remove the free azido-Q, and detergent-azido Q adducts from the protein. The organic solvent remaining in the aqueous layer was evaporated under a stream of nitrogen gas before the solution containing the photolyzed protein was subjected to lyophilization. The lyophilized sample was dissolved in 20 mM Tris-Cl, pH 7.0, containing 1% SDS and 1% β-mercaptoethanol and the solution was incubated at 37 °C for 2 hrs before being subjected to preparative SDS PAGE. The SDS PAGE gel was prepared according to Schägger *et al.*(69) except that 7 M urea was used in

the separating gel instead of 13% glycerol. For the reference wells, the digested sample was treated with fluorescamine prior to sample loading. The electrophoresis was run at 15 V for 2 hrs and then at 45 V for another 15 hrs. The protein bands were visualized by fluorescence under UV. The SDS PAGE pattern of the fluorescamine-treated sample was identical to that of the untreated sample stained by Coomassie Blue. The SdhC protein band was excised from the SDS PAGE gel, and the protein was eluted with an electroeluter from Bio-Rad.

Protease K Digestion of [^{3}H]Azido-Q-labeled SdhC-- Purified [^{3}H]azido-Q-labeled SdhC obtained by electroelution was subjected to a repeated dilution and concentration process using Centricon-10 with dilution buffer of 30 mM Tris-Cl, pH 7.5. The final protein concentration was about 2 mg/ml, with the SDS concentration around 0.5%. The protein was then digested with protease K at 37 °C for 6 hrs using a protease K:SdhC ratio of 1:50 (w/w).

Isolation of Ubiquinone-Binding Peptides-- 100-µl aliquots of the protease K-digested SdhC were separated by high performance liquid chromatography (HPLC) on a Synchropak RP-8 column (0.46 x25 cm) using a gradient formed from 0.1% trifluoroacetic acid and 90% acetonitrile containing 0.1% trifluoroacetic acid with a flow rate of 0.8 ml/min. 0.8-ml fractions were collected. The absorbance at 214 nm and the radioactivity of each fraction were measured. Peaks with high specific radioactivity were collected, dried, and subjected to peptide sequence analysis.

Other Biochemical Methods-- The content of cytochrome b_{556} was determined from the reduced-minus-oxidized spectrum using the millimolar extinction coefficient of 22.8 mM^{-1} cm^{-1} for the wavelength pair 558-575 nm as previously described (42). Absorption spectra were recorded with a Shimadzu spectrophotometer, model UV2101PC. The protein centration was determined by Lowry method (71).

RESULTS AND DISCUSSION

Identification of Q-binding Subunit in Succinate-Q Reductase by Photoaffinity Labeling with [^{3}H]azido-Q Derivatives-- Study of the Q:protein interactions in succinate-Q reductase using synthetic Q derivatives requires the prior removal of endogenous Q from the complex because the binding affinity of synthetic Q derivatives to the Q-binding proteins (sites) is weaker than that of endogenous Q. Since purified *E. coli* succinate-Q reductase contains less than 5% of endogenous Q_{10} with respect to FAD (36) and is fully active upon addition of exogenous Q, it has become an ideal system for studying Q:protein interactions and for identifying the Q-binding site in the succinate-Q reductase with azido-Q derivatives.

When purified succinate-Q reductase was incubated with 5 molar excess of 3-azido-2-methyl-5-methoxy[^{3}H]-6-geranyl-1,4-benzoquinone ([^{3}H]azido-Q) or 5-azido-2,3-dimethoxy[^{3}H]-6-geranyl-1,4-benzoquinone ([^{3}H]-5-azido-Q) for 10 minutes at 0 °C in the dark and then illuminated with long wavelength UV light for 7 minutes, only the [^{3}H]azido-Q-treated sample showed inactivation and radioactivity uptake by protein, indicating that [^{3}H]azido-Q is suitable for studying the Q:protein interaction in this reductase. This [^{3}H]azido-Q has been successfully used in identification of Q-binding sites in the bc_1 complex from various sources (62, 72) and in mitochondrial succinate-Q reductase (1).

Fig. 8 shows that when succinate-Q reductase was incubated with various concentrations of [^{3}H]azido-Q and illuminated with UV light, , the activity decreased as the concentration of azido-Q was increased. Maximum inactivation of 35% was obtained when 5 mole of [^{3}H]azido-Q per mole of cytochrome b_{556} was used. Inactivation was not due to the inhibition of succinate-Q reductase by photolyzed products of azido-Q because when azido-Q was photolyzed in the absence of reductase and then mixed with succinate-Q reductase, no inhibition was observed. Inactivation can be prevented by pre-incubating the enzyme with 5 molar excess of native Q_2. Inactivation was also not due to

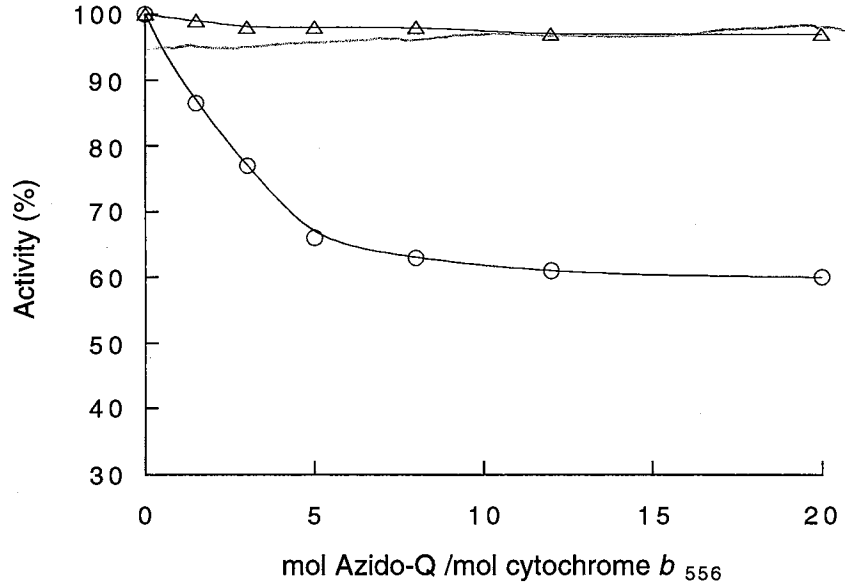


Fig. 8. Effect of azido-Q concentration on succinate-Q reductase activity after illumination. Aliquots (0.2 ml) of succinate-Q reductase from *E. coli*, 2 mg/ml (10.6 μM cytochrome b_{556}), in 50 mM phosphate buffer, pH 7.5, 0.1% E9C12 were mixed with 3 μl of an alcoholic solution containing indicated concentrations of azido-Q derivative, in the dark. After incubation at 0 °C for 10 minutes, the samples were illuminated for 7 min at 0 °C. Succinate-Q reductase activity was assayed before (Δ) and after (o) illumination. 100% activity is succinate-Q reductase without treatment with azido-Q and without illumination.

damage of the reductase protein by UV because when the enzyme alone was illuminated with UV light under the same conditions, no activity loss was observed. These, together with the finding that the amount of azido-Q uptake by succinate-Q reductase correlated with the extent of the enzymatic inactivation (about 0.35 moles of azido-Q uptake per mole of cytochrome b_{556} at 35% inactivation) indicate that inactivation results from the binding of azido-Q to the ubiquinone binding site. Since purified *E. coli* succinate-Q reductase contains less than 5% of endogenous Q, we were rather surprised to see a maximum uptake of 0.35 moles of azido-Q per mole of cytochrome b_{556}. This low azido-Q uptake may result from some the Q-binding sites in the enzyme complex being partially masked by detergent E9C12.

Fig. 9 shows the [^{3}H] radioactivity distribution among subunits of succinate-Q reductase. When the illuminated, [^{3}H]azido-Q-treated succinate-Q reductase was extracted with organic solvent to remove the non-protein bound azido-Q, such as free azido-Q, or detergent-azido-Q adduct and subjected to SDS PAGE, radioactivity was found only in the SdhC subunit, suggesting that this subunit provides the Q-binding site in this enzyme complex. The lack of azido-Q labeling in the SdhD subunit is in contrast to result with the mitochondrial enzyme in which radioactivity was found in all the membrane-anchoring subunits, QPs1 (50%), QPs2 (22%), and QPs3 (25%). It should be noted that when the azido-Q concentration used is higher than a 5 molar excess to cytochrome b_{556}, non-specific labeling of the Fp and Ip subunits is observed because the labeling did not cause the activity to further decrease.

Isolation and Characterization of Ubiquinone-Binding Peptides of SdhC-- In order to identify the Q-binding domain in SdhC through isolation and sequencing of an azido-Q-linked peptide, it is absolutely necessary that the isolated azido-Q-labeled SdhC be free from contamination with unbound azido-Q and completely susceptible to proteolytic enzyme digestion. [^{3}H]azido-Q-labeled SdhC was isolated from illuminated, [^{3}H]-azido-Q treated succinate-Q reductase by a procedure involving ammonium sulfate fractionation,

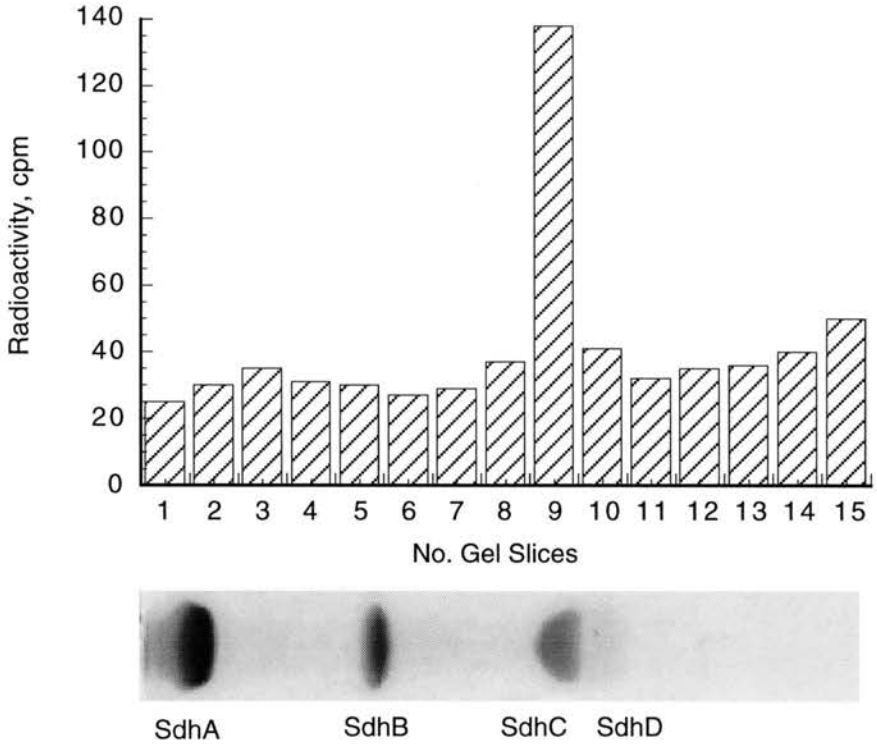


Fig. 9. [^{3}H] radioactivity distribution among subunits of succinate-Q reductase. Purified succinate-Q reductase was treated with [^{3}H]azido-Q at a ratio of 5 mol [^{3}H]azido-Q/mol of cytochrome b_{556} in the dark for 10 min and illuminated for 7 min at 0 °C. The illuminated sample was subjected to organic solvent extraction as described in "Experimental Procedure" to remove all the non-protein bound [^{3}H]azido-Q, and lyophilized. The lyophilized sample was digested with 1% SDS and 1% β-mercaptoethanol at 37 °C for 2 hrs before being applied to an SDS PAGE gel. The electrophoretic conditions were described in "Experimental Procedure". The protein bands were visualized by fluorescence under UV and sliced. The portion containing no protein was also sliced to the same size as that of protein bands. The gel slices were smashed and mixed with 5 ml of Insta-gel and the radioactivity was determined.

dialysis, organic solvent extraction, lyophilization, preparative SDS PAGE, electrophoretic elution, and membrane concentration. Two steps in the isolation procedure, organic solvent extraction and SDS PAGE, are used to remove non-protein bound azido-Q adducts. The SDS concentration in the final purification step is about 0.5% while the concentration of [^{3}H]azido-Q-labeled SdhC is about 2 mg/ml. Isolated [^{3}H]azido-Q-labeled SdhC shows only one band in SDS PAGE corresponding to the third subunit of succinate-Q reductase. About 40% of the SdhC protein present in succinate-Q reductase was recovered in the final purification step, assuming that the molecular mass of succinate-Q reductase is 117 kDa and that it contains one mole of SdhC/mole of reductase. This relatively low yield of SdhC is probably due to very thin slicing of the SdhC band from SDS PAGE gel to avoid contamination with SdhD protein.

When the SDS present in the purified [^{3}H]azido-Q-labeled SdhC was removed by cold acetone precipitation, the resulting protein is highly aggregated and resistant to proteolytic enzyme digestion. Inclusion of 0.1% SDS and 2 M urea in the digestion mixture does not increase proteolysis. Modification of SDS-free, [^{3}H]azido-Q-labeled SdhC by reductive carboxymethylation followed by succinylation also does not render the protein susceptible to chymotrypsin or trypsin digestion. This is different from the mitochondrial system in which reductive carboxymethylation and succinylation effectively rendered purified, SDS-free, [^{3}H]azido-Q-labeled QPs1 and QPs3 susceptible to chymotrypsin or trypsin digestion.

Since the SDS-free [^{3}H]azido-Q-labeled SdhC, with or without prior chemical modification, cannot be completely digested by proteolytic enzymes, an alternative way is to digest isolated [^{3}H]azido-Q-labeled SdhC with a protease that is active even when SDS concentration is higher than 0.5%. Of the commercially available proteolytic enzymes, only protease K was reported to be active in 0.5% SDS and 1 M urea. Therefore, isolated [^{3}H]azido-Q-labeled SdhC was subjected to protease K digestion at 37 °C using a protease K/SdhC ratio of 1:50 (w/w). To obtain the optimal digestion time 100-µl of aliquots were

withdrawn from the digestion mixture at different time intervals, subjected to HPLC separation, and analyzed for peptide patterns and radioactivity recovery on HPLC chromatograms. A 6-hr digestion time was found to be optimal. Fig. 10 shows the ^{3}H radioactivity distribution on HPLC chromatograms of [^{3}H]azido-Q-labeled SdhC digested with protease K for 6 hrs at 37 °C. Most of the radioactivity was found in a fraction with a retention time of 61.7 min (P-61.7). The radioactivity recovery is about 65% based on the radioactivity applied to the HPLC column.

The partial NH_2-terminal amino acid sequence of P-61.7 was found to be TIRFPITAIASILHRVS-, corresponding to amino acid residues 17-33 of SdhC. Judging from the gradually decreased amino acid recoveries in sequencing chromatograms, this peptide probably ends at residue 36. The Q-binding domain, using the proposed structure of SdhC (see Fig. 11), is located before the start of transmembrane helix I, toward the cytoplasmic side of the membrane. The proposed structural model of SdhC, shown in Fig. 11, is a modification of the one from Hägerhäll *et al.* (39) with Arg-31 remaining in the cytoplasm instead of being in the transmembrane helix I. This modification is based on thermodynamic considerations. In this Q-binding region are several highly conserved amino acid residues for type C anchors of succinate-Q reductase, such as Ser-27, His-30, Arg-31, and Ser-33. It should be noted that the Q-binding domain identified in *E. coli* SdhC is different from that identified in bovine heart mitochondrial QPs1 (1).

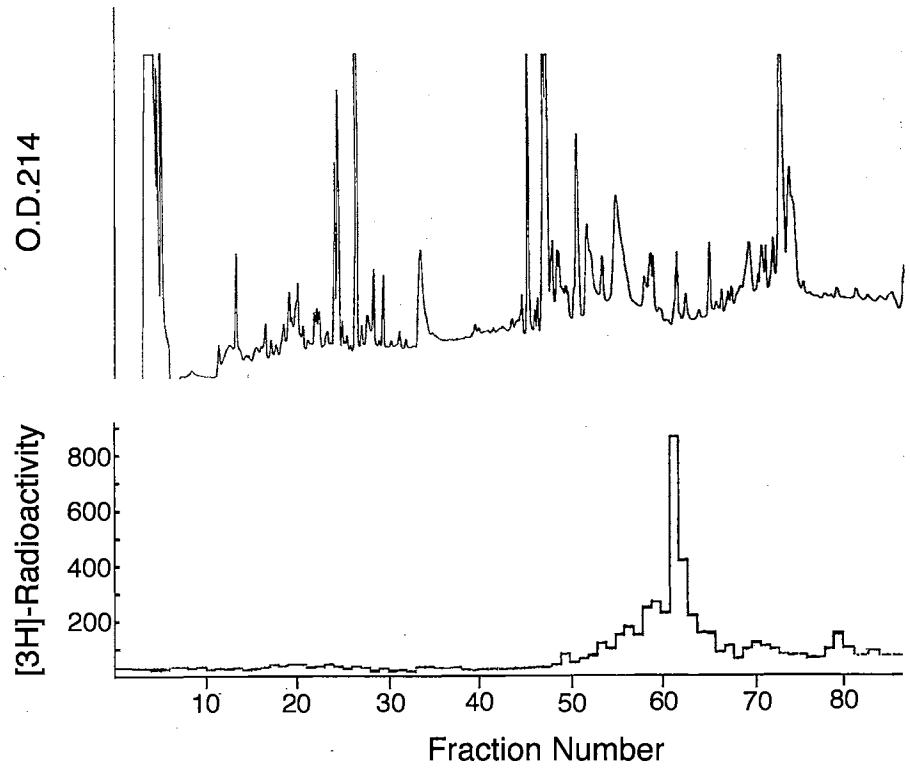


Fig. 10. ^{3}H radioactivity distribution in an HPLC chromatogram of protease K-digested, [^{3}H]azido-Q-labeled SdhC protein. The [^{3}H]azido-Q-labeled SdhC (2 mg/ml, 1x10^{5} cpm/mg) was mixed with protease K, and 100 µl aliquots of digested solution were subjected to HPLC separation as described in "Experimental Procedure". 100 µl aliquots were withdrawn from each fraction for radioactivity determination.

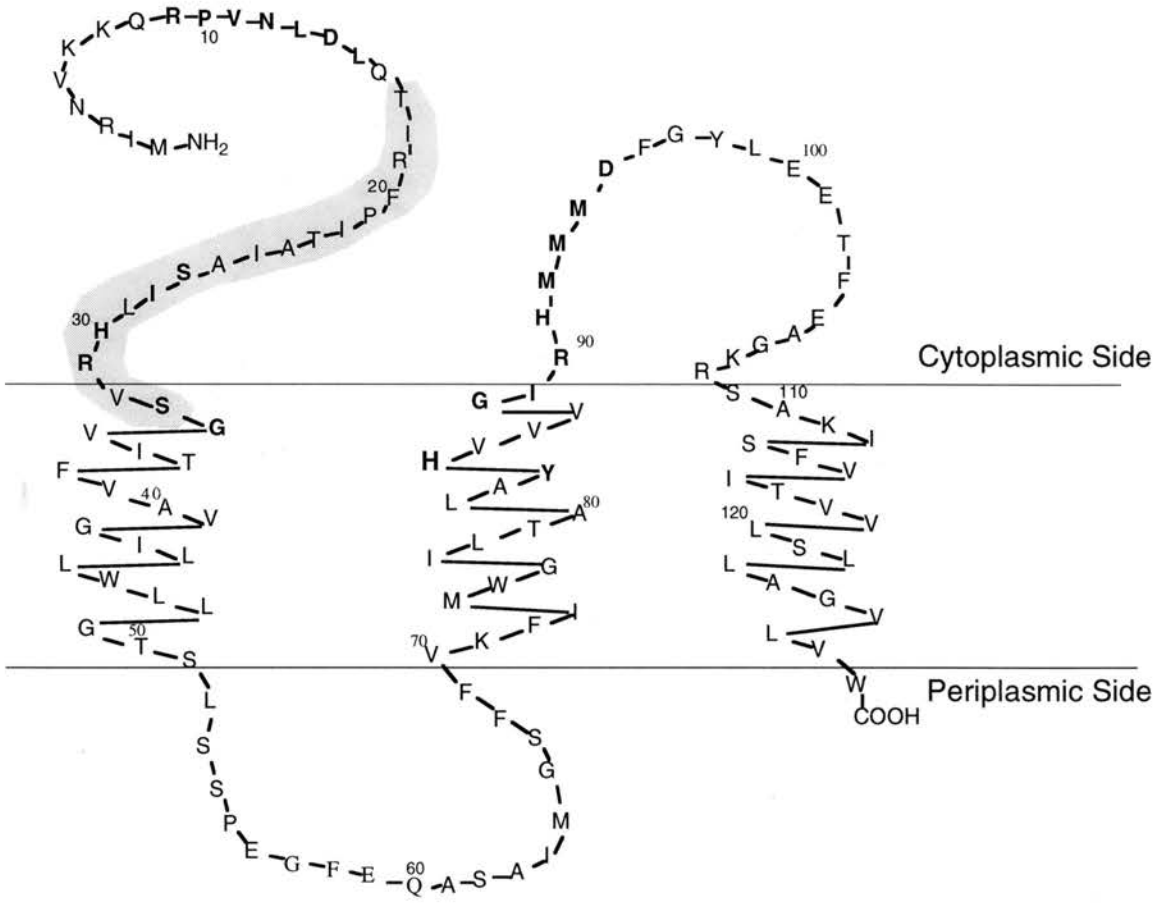


Fig. 11. Proposed secondary structure of SdhC. The shaded area is the Q-binding peptide identified in this report. Bold face letters represent the conserved amino acid residues of type C anchors. This structure is a modification of the one proposed by Hägerhäll, C., and Hederstedt, L., with Arg-31 being exposed to the cytoplasm instead of buried inside the transmembrane helix I.

CHAPTER IV

DELETION OF THE *SDH* OPERON FROM *E. COLI* CHROMOSOME

SUMMARY

A *E. coli* strain lacking genomic succinate-Q reductase (ΔSQR) is generated by homologous recombination. A kanamycin resistant gene flanked by homologous regions to *E. coli sdh* operon on a pSELECT-1 plasmid (ΔSQR::kn^R/pSELECT-1) is transformed into *E. coli* JC7623. Colonies with double-crossover events are selected for Kn^R and Tc^s phenotypes. An expression strain NM256 (ΔSQR::kn^R) is constructed by P1 transduction from strain JC7623 (ΔSQR:: kn^R) into NM256 (*recA*). The deletion of the *sdh* operon is confirmed by medium selection, PCR reaction, and complementation studies.

INTRODUCTION

After localization of the Q-binding domain in SdhC by photoaffinity labeling, it is necessary to identify the amino acid residues in this region responsible for Q-binding in order to fully understand the Q-binding site. Since the *sdh* operon has been cloned, sequenced (76), and over-expressed in *E. coli* NM256 strain (42), an effective and unambiguous way to attack this problem is by site-directed mutagenesis of the *sdhC* gene in the *sdh* operon followed by complementation of the mutated operon to an *E. coli* strain lacking succinate-Q reductase. To obtain the *E. coli* strain lacking succinate-Q reductase

we chose to delete most of the *sdh* operon by homologous recombination, rather than to inactivate it by insertion or point mutation, in order to avoid possible recombinations.

EXPERIMENTAL PROCEDURES

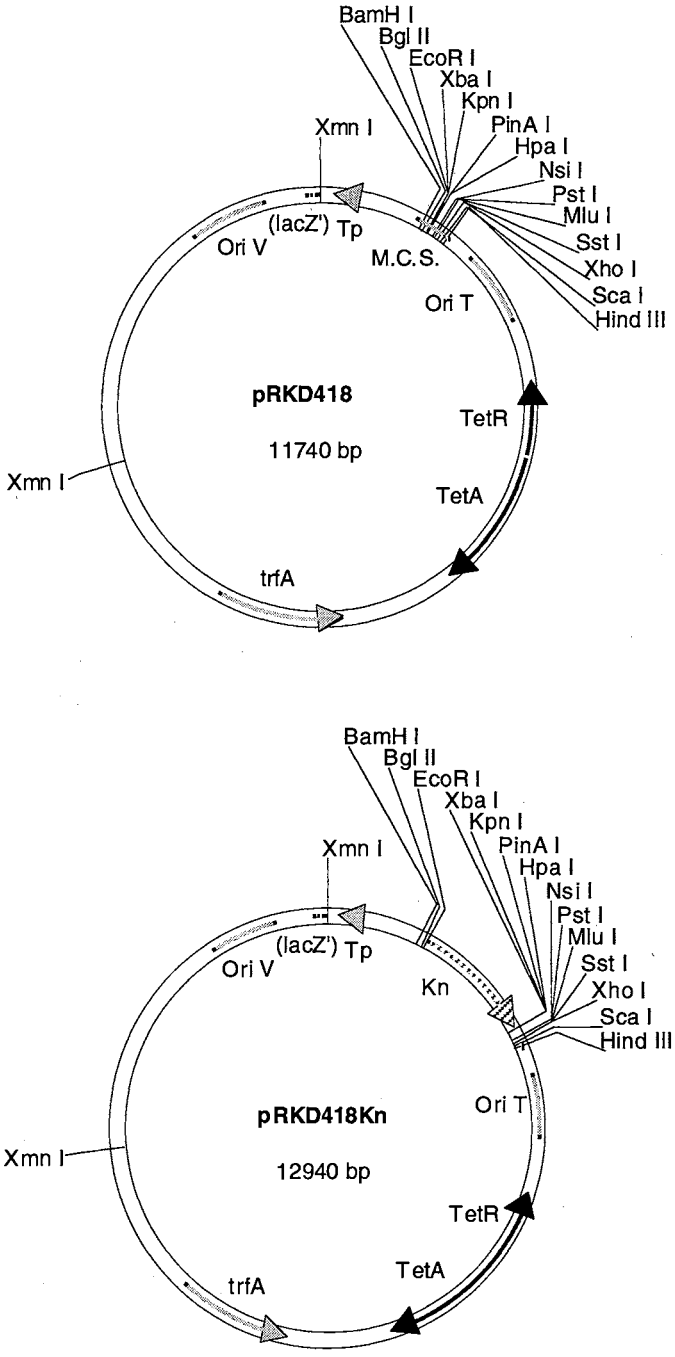
Materials-- pRKD418 (Scheme VIII), which was used for the expression of the *sdh* operon; pSL1180KnERV, which was used to provide the kanamycin resistant gene (Kn^R) were constructed in our laboratory (73). Restriction endonucleases and DNA modifying enzymes were purchased from Promega, Life Technologies, New England Biolabs, and Pharmacia. *E. coli* strain JC7623 (*recB*21 *recC*22 *sbcB*15 *sbcC*201) and phage P1*clr100* were generously provided by Dr. R. Essenberg of Oklahoma State University. *E. coli* strain NM256 (*recA*) was generously provided by Dr. J. R. Guest of the Sheffield University, UK.

sdhCDAB /pRKD418 is constructed by ligation of a 4.5 kb *Eco*RI and *Xba*I fragment including the whole *sdh* operon into the *Eco*RI/*Xba*I sites of pRKD418Kn (see Scheme VIII) (73) which was constructed by insertion of a Kn^R gene between the *Eco*RI/*Xba*I sites of pRKD418. Loss of kanamycin resistance was then used to screen for recombinant plasmids. For complementation studies, *sdhCDAB* /pRKD418 derivative was transformed into the succinate-Q reductase deletion strain, *E. coli* NM256 (*recA*) (EΔSQR).

Growth of Bacteria-- *E. coli* cells were grown at 37 °C in LB medium or M9 minimal medium containing 20 mM succinate and required amino acids with vigorous gyratory shaking. Where appropriate, ampicillin, 100-125 μg/ml; tetracycline, 10-15 μg/ml; kanamycin sulfate, 30-50 μg/ml; or trimethoprim, 85-100 μg/ml was used for the growth of *E. coli*.

Other Recombinant DNA Techniques-- General molecular genetic manipulations were performed essentially as described by Sambrook *et al.* (74). P1 transduction was

Scheme VIII. Maps of vectors used for the expression of *sdh* operon.



performed as described by Miller (75).

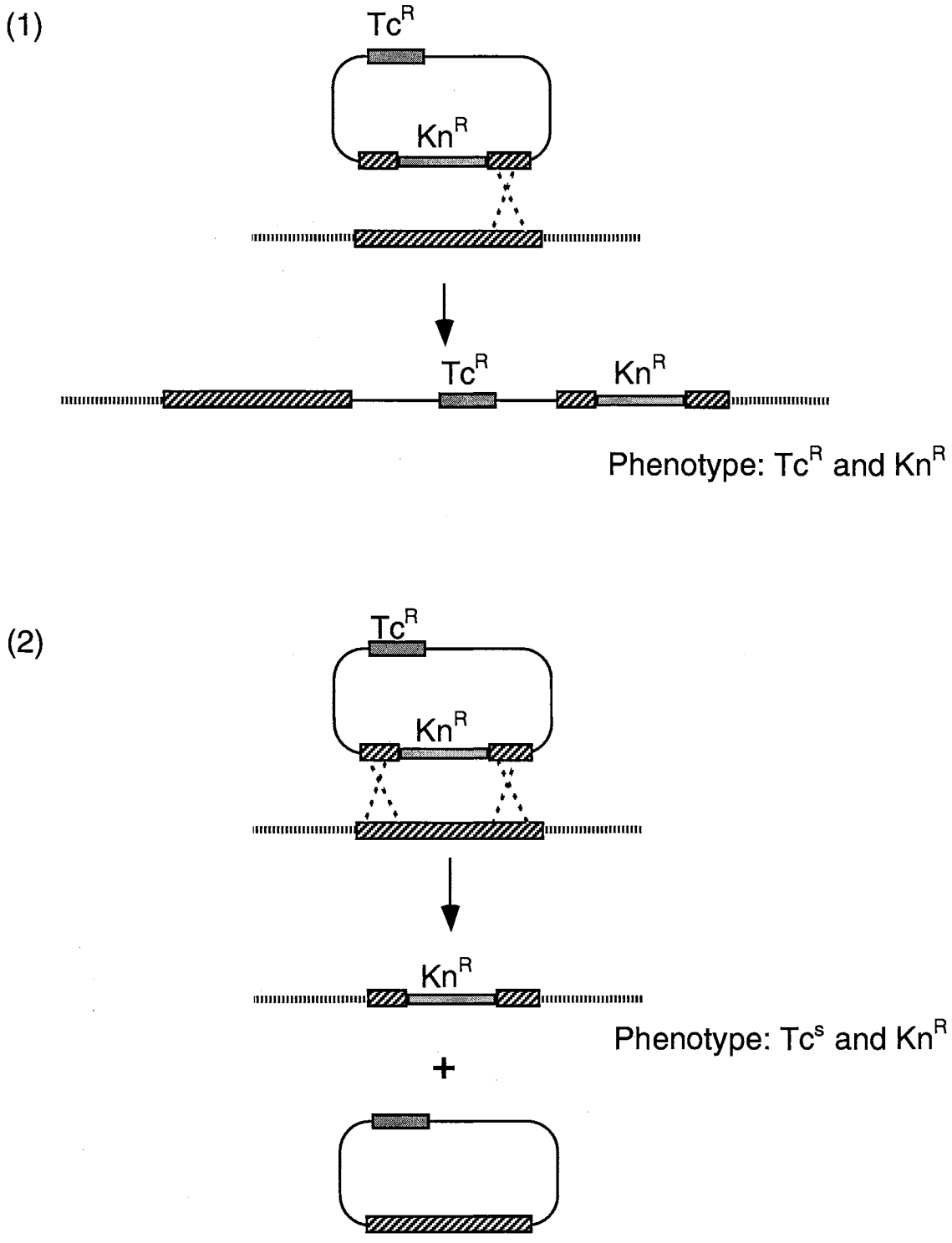
Membrane Preparations and Activity Assays-- Membranes were prepared from NM256 and EΔSQR strains as described previously (42). Succinate-Q reductase activity was assayed for its ability to catalyze Q-stimulated DCPIP reduction by succinate. The assay was performed at room temperature in a Shimadzu UV2101PC spectrophotometer. About 0.5 μg of enzyme was added to an assay mixture (1 ml) containing 50 mM sodium/potassium phosphate, pH 7.0, 50 μM DCPIP, 20 mM succinate, 1 mM EDTA, 0.01% Triton X-100 and 25 μM of Q_2. The reduction of DCPIP was followed by measuring the absorbance decrease at 600 nm, using a millimolar extinction coefficient of 21 mM^{-1} cm^{-1}.

RESULTS AND DISCUSSION

Deletion of the *sdh* Operon from *E. coli* Chromosome.--Scheme IX describes the strategy used in this study for the genomic replacement of the *sdh* genes from *E. coli* by homologous recombination. *E. coli* is transformed with a plasmid which contains a Kn^R gene segment flanked by *sdh* seuqences homologous to regions on the *E. coli* chromosome. Since this special *E. coli* strain can not stabilize the plasmid (see Result and Discussion), under the selection pressures (Kn^R), cells which integrate the plasmid into the chromosome or exchange DNA with the plasmid through homologous recombination will be at an advantage. A single-crossover event results in the integration of the plasmid into the chromosome, and these cells exhibit both Kn^R and Tc^R phenotype; while a double-crossover event will result in the replacement of most of the *sdh* genes from the chromosome by Kn^R gene. The cells will be Kn resistant but Tc sensitive.

Fig. 12 summarizes the procedure for genomic replacement of the most of the *sdh* operon with a Kn^R gene in *E. coli* NM256. A 4.5 kb *Bam*HI fragment containing the *sdh* operon from pGS133 plasmid was ligated into the *Bam*HI site of pSELECT-1 to generate

Scheme IX. The strategy used in this study for the genomic replacement of the *sdh* genes from *E. coli* by homologous recombination.



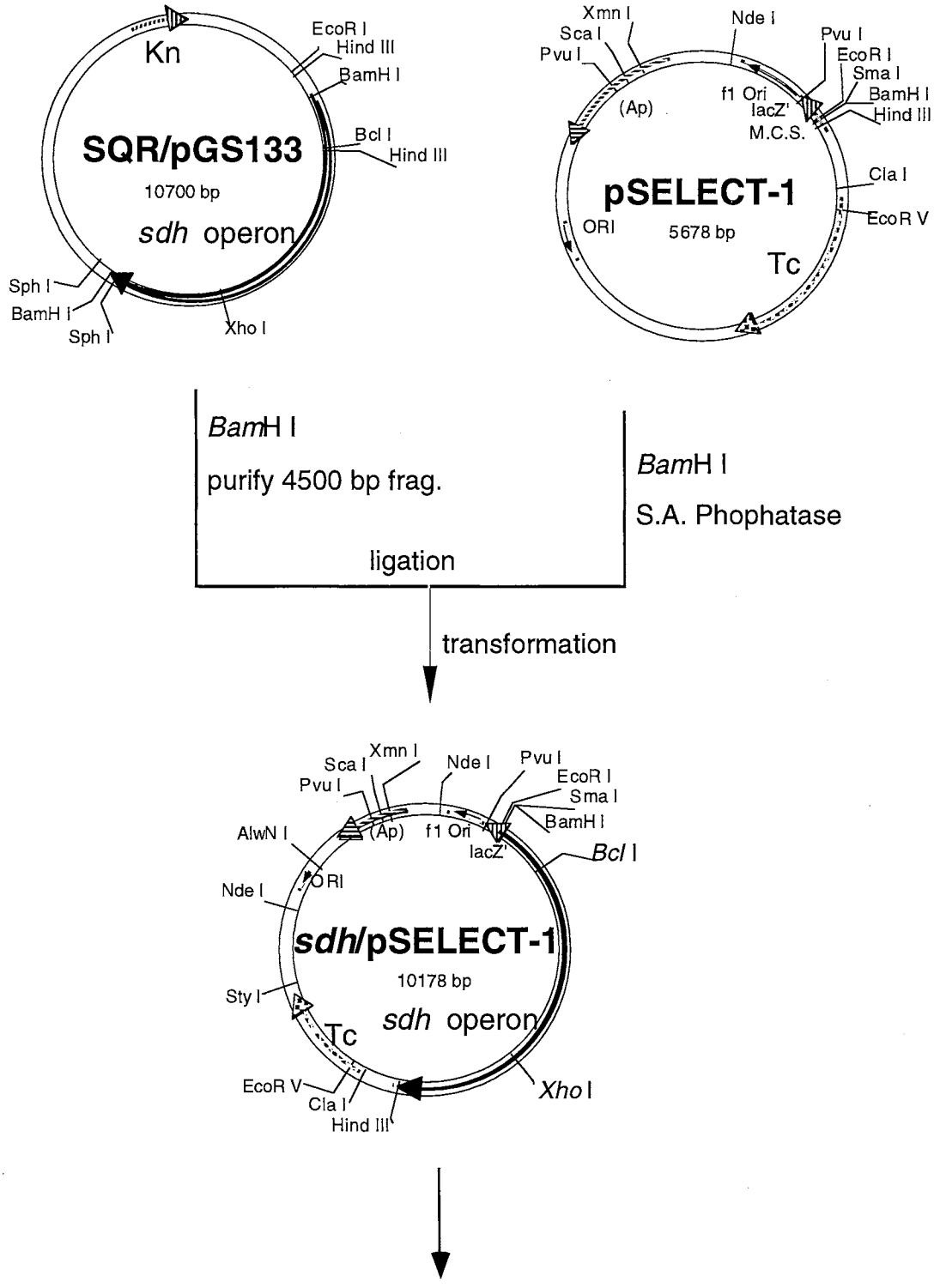
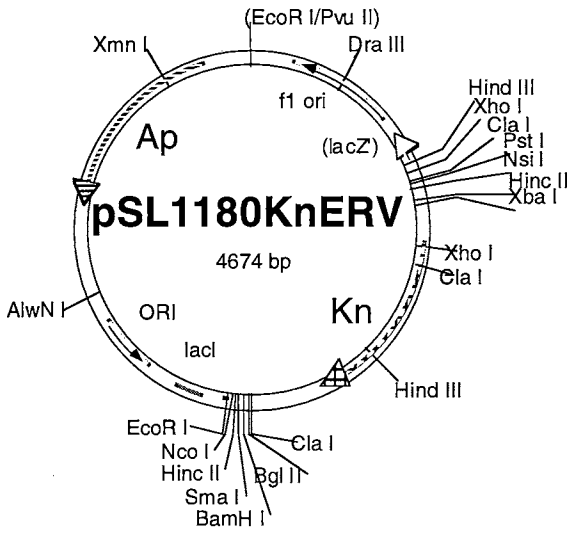


Fig. 12. Protocol for the construction of *E. coli* NM256 lacking succinate-Q reductase (EΔSQR).

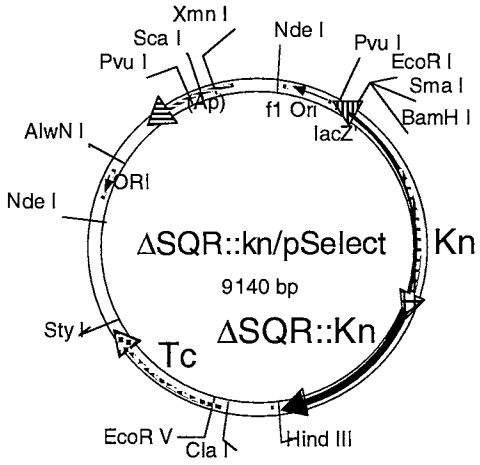


sdh/pSELECT-1
(from previous page)

Bcl I
Xho I
Klenow fill-in
S.A. Phophatase
ligation

Hinc II
Purify 1380 bp frag.

Transformation (select Kn^R and Tc^R)



Electroporation into JC7623
Select Kn^R and Tc^S

JC7623 (ΔSQR::Kn^R)

P1 transduction into NM256(*rec*A)

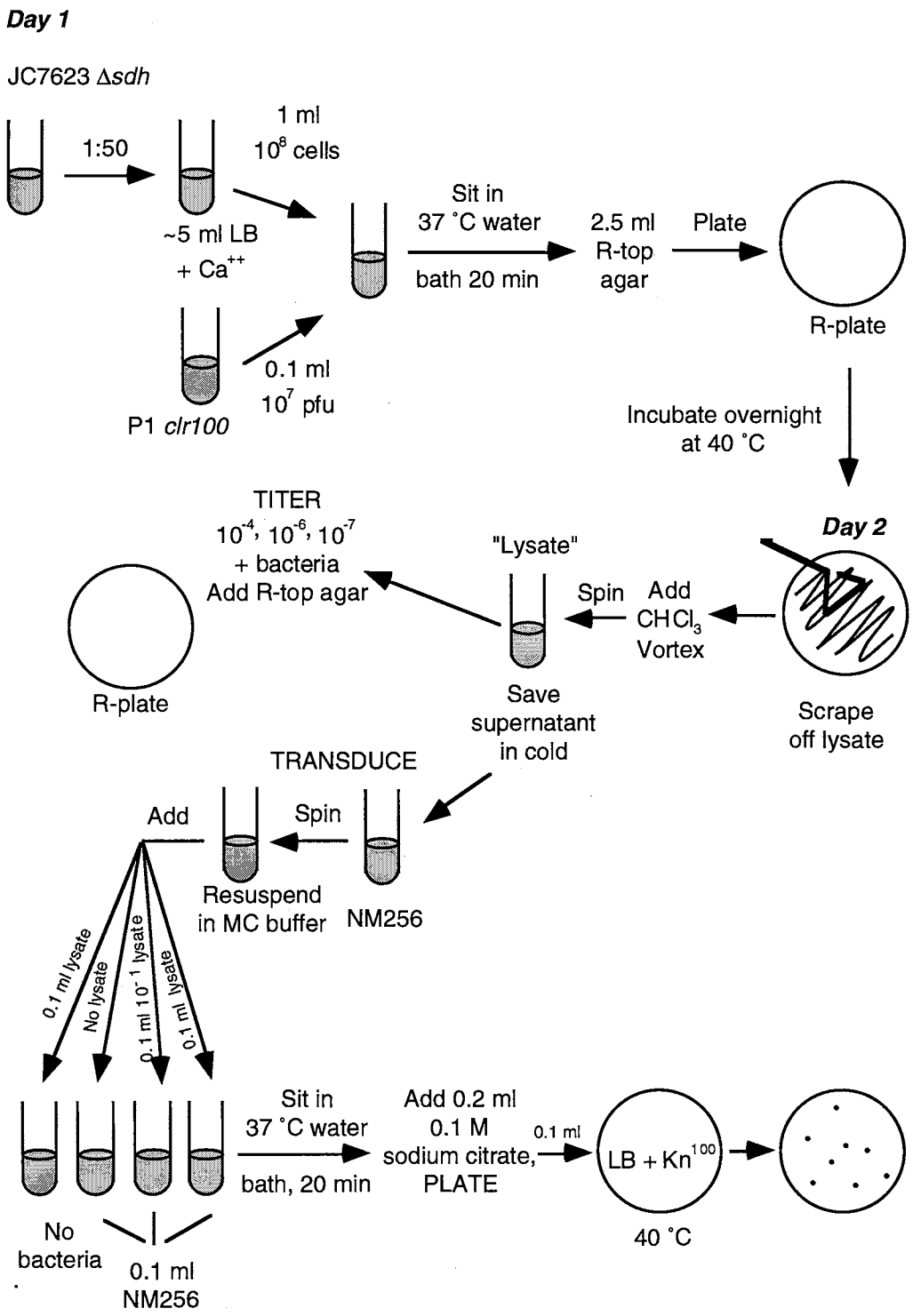
NM256 (ΔSQR::Kn^R, *rec*A)
(EΔSQR)

sdh /pSELECT plasmid. The cloning of the *sdh* operon into the pSELECT-1 plasmid enables us to introduce a Kn^R gene into the resulting plasmid because it contains no Kn^R gene. The *sdh* /pSELECT plasmid was digested with *Bcl*I and *Xho*I to remove *sdhC*, *D*, *A*, and the beginning of *sdhB*. The remaining linear fragment was then treated with Klenow fragment to generate blunt end and ligated with a 1.2 kb Kn^R gene cassette contained in *Hin*cII fragment from pSL1180KnERV plasmid. The resulting plasmid, Δ*sdh*::Kn^R/pSELECT was electroporated into *E. coli* JC7623 and cells were selected for Kn^R and Tc^S phenotypes. It has been shown that replication of ColEI-derived plasmids in *recBC sbcBC* strains is deleterious to cell growth due to the formation of linear multimers (77). Under conditions where maintenance of a plasmid is selected, cells in which the plasmid has integrated would be at an advantage. It has also been reported by Oden that in this strain covalently-closed-circular (ccc) plasmid DNA is at least as effective for gene replacement by transformation as is linearized plasmid DNA (78). Thus cells with the Kn^R and Tc^S phenotypes must result from replacement of the *sdh* operon by Kn^R gene in chromosomes arising from a double-crossover event between circular plasmid DNA and chromosomal DNA.

The Kn^R-Tc^S cells (JCΔSQR::Kn^R) are unable to grown on M9/succinate medium. However, since JCΔSQR::Kn^R cells are *recA*$^+$, they cannot stably harbor plasmid and thus cannot be used for expression of cloned *sdh* in a plasmid. The Kn^R in the chromosome of JCΔSQR::Kn^R cells was introduced into *E. coli* NM256 (*recA*) by P1 transduction using P1*clr100* (Scheme X). The resulting NM256 with Kn^R phenotype is named EΔSQR.

The replacement of the *sdh* operon with the Kn^R gene in EΔSQR cells was confirmed by PCR reaction, medium selection, and gene complementation. When genomic DNA was extracted from EΔSQR and used as template for PCR amplification using a forward primer, AAAATCTCCTTTGTTATTACTG-3', which is located at the end of the *sdhC* gene and a reverse primer, AATTCTCTGACTGGCAATTTCA-3', which is located at the beginning of *sdhA*, no PCR product was obtained. However, when genomic DNA

Scheme X. Flow chart for the preparation and use of P1 *clr100* lysates.



prepared from the wild-type cell was used as template, a 420-bp PCR product was obtained, confirming that most of the *sdh* genes are absent from the chromosome of EΔSQR cells (Fig 13).

EΔSQR cells are unable to support aerobic growth on M9 medium supplemented with essential amino acids using succinate as carbon source (M9/succinate medium). To confirm that the failure of EΔSQR to grow aerobically in M9/succinate medium is due to deletion of the *sdh* operon, the intact *sdh* operon contained in a 4.5 kb *Bam*HI fragment on a broad range, low copy number plasmid pRKD418 was transformed into the cell. The resulting strain (complement) grew aerobically in M9/succinate medium at a rate similar to that of a wild-type strain harboring the pRKD418. pRKD418 plasmid carries tetracycline and trimethoprim (Tp) resistant genes (73) while EΔSQR carries kanamycin resistant gene. Thus transformants are selected for Tp^R, Tc^R and Kn^R phenotypes. It should be mentioned that pGS133, a low copy number plasmid which was used previously to over-express succinate-Q reductase in *E. coli* NM256 is not suitable for use in complementation of the *sdh* operon in EΔSQR cells because the plasmid carries the same antibiotic resistant gene, Kn^R, as that in the chromosome of EΔSQR cell and thus makes selection of transformants difficult. The yield and activity of succinate-Q reductase obtained from EΔSQR harboring cloned *sdh* operon in pRKD418 are comparable to those of succinate-Q reductase obtained from *E. coli* NM256 containing cloned *sdh* operon in pGS133.

Although EΔSQR cells cannot grow aerobically in M9/succinate medium, they are able to support aerobic growth in LB medium at a rate slower than that of wild-type cells. No succinate-Q reductase activity was detected in membrane preparations obtained from EΔSQR cells grown aerobically in LB medium, indicating that *E. coli* can rely on various dehydrogenases to support aerobic growth.

Table IV summarizes the phenotypic differences between strains EΔSQR and NM256.

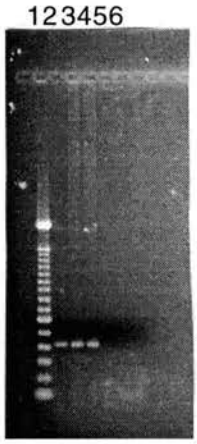


Fig. 13. PCR reactions on the genomic DNA from NM256 and EΔSQR cells with described primers. Lane 1, DNA ladder; lane 2-4, NM256 cells with Kn^R and Tc^R phenotype; lane 5, 6, EΔSQR cells with Kn^R and Tc^s phenotype.

TABLE IV. COMPARISON OF PHENOTYPES OF STRAINS EΔSQR AND NM256

	EΔSQR strain (*sdh*$^-$)	NM256 strain (*sdh*$^+$)
Growth rate on succinate	–[a]	++++[b]
Growth rate on LB	++	++++
SQR activity in membrane preparation	–	~2.5 μmol succinate oxidized/min/nmol b_{556}
Detection of cytochrome b_{556} in membrane	–	+
Growth on succinate after complementation with cloned *sdh* operon	++++	N/A[c]

a, – indicated no growth on succinate within 5 days.

b, ++++ indicated that the growth rate is essentially the same as the "wild type".

c, N/A, not applicable.

CHAPTER V

IDENTIFICATION OF AMINO ACID RESIDUES INVOLVED IN Q-BINDING OF SDHC

SUMMARY

To identify amino acid residues responsible for ubiquinone-binding substitution mutations at the putative ubiquinone-binding region of SdhC were generated and characterized. *E. coli* NM256 lacking genomic succinate-Q reductase genes was used to harbor the mutated succinate-Q reductase genes in a low copy number pRKD418 plasmid. Substitution of Serine-27 of SdhC with alanine, cysteine, or threonine, or of Arginine-31 with alanine, lysine, or histidine, yields cells unable to grow aerobically in minimum medium with succinate as carbon source. Furthermore, little succinate-ubiquinone reductase activity or [^{3}H]azido-Q uptake is detected in succinate-ubiquinone reductases prepared from these mutant cells grown aerobically in LB medium. These results indicate that both the hydroxyl group and the size of the amino acid side chain at position 27 , and the guanidino group at position 31 of SdhC are critical for succinate-ubiquinone reductase activity, perhaps by formation of hydrogen bonds with carbonyl groups of the 1,4-benzoquinone ring of the quinone molecule. The hydroxyl group, but not the size of the amino acid side chain, at position 33 of SdhC, is also important for succinate-ubiquinone reductase because Ser-33 can be substituted with threonine but not with alanine.

INTRODUCTION

The amino acid residues in the putative Q-binding domain of SdhC essential for Q-binding were investigated by using site-directed mutagenesis coupled with biochemical and biophysical characterizations of succinate-Q reductase obtained from EΔSQR cells carrying cloned *sdh* operon with mutations in the *sdhC* gene in pRKD418. 3-D structural information and site-directed mutagenesis studies (81, 82) of the Q-binding sites in photosynthetic bacterial reaction centers have revealed two categories of amino acid residues involved in Q-binding. One is those having the ability to form hydrogen bonds with the carbonyl groups of the 1,4-benzoquinone ring of quinone, such as His M217 and Ala M258 of *R. viridis* and Thr M220 and Ala M258 of *R. sphaeroides* for the Q_A site, and His L190 and Ser L223 of *R. viridis* for the Q_B site. The other category is amino acid with the ability to stabilize Q-binding through π-π interactions with the benzoquinone ring, such as Phe L216 of *R. viridis* (82).

EXPERIMENTAL PROCEDURES

Materials: Dichlorophenol-indophenol (DCPIP), polyoxyethylene-9-lauryl ether (E9C12), and flavin mononucleotide (FMN) were obtained from Sigma. DEAE-Sepharose CL-6B was from Pharmacia LKB Biotechnology Inc. Other chemicals were of the highest purity commercially available.

Growth of Bacteria-- *E. coli* cells were grown at 37 °C in LB medium or M9 minimal medium containing 20 mM succinate and required amino acids with vigorous gyratory shaking. Cell growth was monitored by measuring the increase of optical density at 660 nm. Where appropriate, ampicillin, 100-125 μg/ml; tetracycline, 10-15 μg/ml; kanamycin sulfate, 30-50 μg/ml; or trimethoprim, 85-100 μg/ml was used for the growth

of the mutants of *E. coli*. Extra rich medium, TYP, was used in the procedures for the rescue of single-stranded DNA (79).

Construction of Mutations and Expression of Mutated Succinate-Q Reductase in *E. coli* -- Mutants were constructed by site-directed mutagenesis using the Altered-Sites system from Promega (80), and mutagenic oligonucleotides were synthesized at the OSU Recombinant DNA/Protein Core Facility. The mutagenic oligonucleotides used are:

CTGGACCTACAGGCCATCCGGTTCCC (T17A),

CTACAGACCATCGCGTTCCCCATCACGGCG (R19A),

CAGACCATCCGGCTCCCCATCACGGCG (F20L),

CGGTTCCCCATCGCGGCGATAGCGT (T23A),

ACGGCGATAGCGGCCATTCTCCATC (S27A),

ACGGCGATAGCGTGCATTCTCCATCG (S27C),

ACGGCGATAGCGACCATTCTCCATCG (S27T),

GCGTCCATTCTCGCTCGCGTTTCCGG (H30A),

TCCATTCTCCATGCCGTTTCCGGTGTG (R31A),

TCCATTCTCCATAAAGTTTCCGGTGTG (R31K),

TCCATTCTCCATCACGTTTCCGGTGTGATC (R31H),

CTCCATCGCGTTGCCGGTGTGATC (S33A),

CTCCATCGCGTTTGCGGTGTGATCACCTTT (S33C),

CTCCATCGCATTACCGGTGTGATC (S33T).

Plasmid *sdhCDAB*/pSELECT-1, in which a 4.5 kb *Bam*HI fragment containing *sdh* operon was inserted into the *Bam*HI site of pSELECT-1, was used as template DNA for mutagenesis.

Following mutagenesis, a 4.5 kb *Eco*RI and *Xba*I fragment including the whole *sdh* operon was ligated into the *Eco*RI/*Xba*I sites of pRKD418Kn (73) which was constructed by insertion of a Kn^R gene between the *Eco*RI/*Xba*I sites of pRKD418. Loss of kanamycin resistance was then used to screen for recombinant plasmids.

sdhCDAB/pRKD418 derivatives were transformed into a succinate-Q reductase deletion strain *E. coli* NM256 (*recA*) (EΔSQR).

Other Recombinant DNA Techniques-- General molecular genetic manipulations were performed essentially as described by Sambrook *et al.* (74). Nucleotide sequencing was performed at the OSU Recombinant DNA/Protein Core Facility with an Applied Biosystems model 373 automatic DNA sequencer.

Enzyme Preparations and Assays-- Membranes were prepared from EΔSQR complement and mutant strains as described previously (42). The succinate-Q reductase was purified from membranes by the method of Yang *et al.* (36). Succinate-Q reductase activity was assayed for its ability to catalyze Q-stimulated DCPIP reduction by succinate. The assay was performed at room temperature in a Shimadzu UV2101PC spectrophotometer. About 0.5 μg of enzyme was added to an assay mixture (1 ml) containing 50 mM sodium/potassium phosphate, pH 7.0, 50 μM DCPIP, 20 mM succinate, 1 mM EDTA, 0.01% Triton X-100 and 25 μM of Q_2. For determination of apparent K_m for Q_2, various concentrations of Q_2 were used. The reduction of DCPIP was followed by measuring the absorbance decrease at 600 nm, using a millimolar extinction coefficient of 21 mM^{-1} cm^{-1}.

Other Biochemical Methods-- The content of cytochrome b_{556} was determined from the reduced-minus-oxidized spectrum using the millimolar extinction coefficient of 22.8 mM^{-1} cm^{-1} for the wavelength pair 558-575 nm as previously described (42). Absorption spectra were recorded with a Shimadzu spectrophotometer, model UV2101PC.

The FAD contents in wild-type and mutant *E. coli* succinate-Q reductases were determined fluometrically as previously reported (19). *E. coli* SQR sample, containing about 4 nmol of cytochrome b_{556}, was mixed with 50% trichloroacetic acid to give a final concentration of 10 %. The mixture was allowed to stand at 0 °C for 30 min, and then centrifuged. The residue was washed with 1 % trichloroacetic acid and finally suspended in 50 mM phosphate buffer, pH 7.4. The pH of the suspension was adjusted to 7.6 with

2.5 N NaOH and made up to a final volume of 6 ml with 50 mM phosphate buffer. The suspension was digested with trypsin for 4 hrs at 38 °C at the ratio of 1 mg trypsin/mg protein. At the end of the digestion, 50 % trichloroacetic acid was added to the mixture to give a final concentration 9 %. The sample was then incubated overnight at 38 °C. After hydrolysis the samples were diluted to 6 ml and the precipitate was removed by centrifugation. A Perkin-Elmer spectrofluorometer was used for fluorescent measurements. The excitation wave length was 450 nm and the fluorescent (emission) wave length was 520 nm. The slit widths of the excitation monochrometer were 5 nm while those of the fluorescence monochrometer were 10 nm. The fluorescence of each flavin sample was measured before and after reduction by dithionite at three levels between 2 X 10^{-2} μM and 6 X 10^{-2} μM in 30 mM citrate buffer, pH 3.3, and between 6 X 10^{-2} and 1.5 X 10^{-1} μM in 30 mM phosphate buffer, pH 7.4. The standards were subjected to exactly the same measurements and consisted of five levels of free FMN. The net fluorescence intensity (oxidized minus dithionite-reduced) of both the standards and the samples varied linearly with their concentrations. The correction of the fluorescence at pH 7.4 was negligible. The acid-nonextractable flavin was calculated by the equation:

Concentration of acid-nonextractable flavin = (equivalent concentration of FMN at pH 3.2) – (equivalent concentration of FMN at pH 7.4)

The concentration of free FMN standard was determined spectrophotometrically (19) from the reduced-minus-oxidized spectrum using the extinction coefficient of 10.3 mM^{-1} cm^{-1} for the wavelength pair 450-553 nm.

RESULTS AND DISCUSSION

Identification of Amino Acid Residues Involved in Q-binding of SdhC--Assuming that the Q-binding site in SdhC of *E. coli* succinate-Q reductase resembles the one in the photosynthetic bacterial reaction center, Thr-17, Thr-23, Ser-27, Ser-33 (through hydroxyl

groups), His-30 (imidazole group), and Arg-19, Arg-31 (guanidino group) may form hydrogen bonds with the carbonyl groups of the 1,4-benzoquinone ring, and Phe-20 may stabilize the 1,4-benzoquinone ring through π-π interaction. Therefore these amino acid residues were selected for mutagenesis. Serine, threonine, histidine, and arginine residues were mutated to alanine in which the methyl group is not capable of forming hydrogen bonds with the carbonyl group of Q. Phenylalanine-20 was replaced with leucine which lacks conjugated ring structure to stabilize the 1,4-benzoquinone ring through π-π interaction.

Table V summarizes the succinate-supported growth behavior of cells expressing the wild-type SQR (complement cells) and the T17A, R19A, F20L, T23A, S27A, S27C, S27T, H30A, R31A, R31K, R31H, S33A, S33C and S33T-substituted SQR mutations, as well as the electron transfer activities and apparent K_ms for Q_2 of purified succinate-Q reductases derived from these recombinant strains. Replacing Thr-17, Arg-19, or His-30 with alanine, or replacing Phe-20 with leucine yields cells capable of aerobic growth in M9/succinate medium at a rate similar to that of the complement cells. Since the electron transfer activities and the apparent K_ms for Q_2 of T17A, R19A, H30A and F20L-substituted succinate-Q reductases are comparable to those of the complement reductase, Thr-17, Arg-19, His-30, and Phe-20 of SdhC are not involved in the Q-binding.

Replacing Thr-23 with alanine yields cells capable of aerobic growth in M9/succinate medium at a rate slightly slower than that of complement strain. The specific activity of T23A-substituted succinate-Q reductase to catalyze electron transfer from succinate to Q, based on μmol succinate oxidized per nmol cytochrome b_{556}, decreased by 40%, while the apparent K_m' for Q_2 of this mutant reductase is the same as that of complement reductase, indicating that Thr-23 of SdhC may not be involved in Q-binding.

The mutant succinate-Q reductases were photoaffinity-labeled with [^{3}H]azido-Q following conditions for the identification of quinone-binding subunit in wild type succinate-Q reductase (Chapter III, page 51). Fig. 14 shows the [^{3}H] radioactivity

TABLE V. CHARACTERIZATION OF THE SDHC MUTATIONS WITHIN THE Q-BINDING DOMAIN

Mutations	Growth on Succinate	Enzymatic Activity *	Molar ratio of FAD/b_{556}	K_m for Q_2 (μM)
wild type	++++[a]	3.15	1.0	10.8
T17A	++++	2.15	1.1	10.2
R19A	++++	2.67	0.9	9.0
F20L	++++	3.30	1.1	9.0
T23A	+++	1.90	0.9	10.8
S27A	–[b]	0.20	0.9	—[c]
S27C	–	0.23	1.0	—
S27T	–	0.21	1.1	—
H30A	++++	2.33	1.1	10.6
R31A	–	0.17	0.9	—
R31H	–	0.17	1.1	—
R31K	–	0.21	1.1	—
S33A	+	1.12	0.9	11.4
S33C	+	1.43	1.0	8.9
S33T	++	2.81	0.9	10.0

a, ++++ indicates that the growth phenotype is essentially the same as the "wild type".

b, – indicates no growth on succinate within 5 days.

c, can not be determined accurately.

* The activity is expressed in μmol succinate oxidized/min/nmol b_{556}

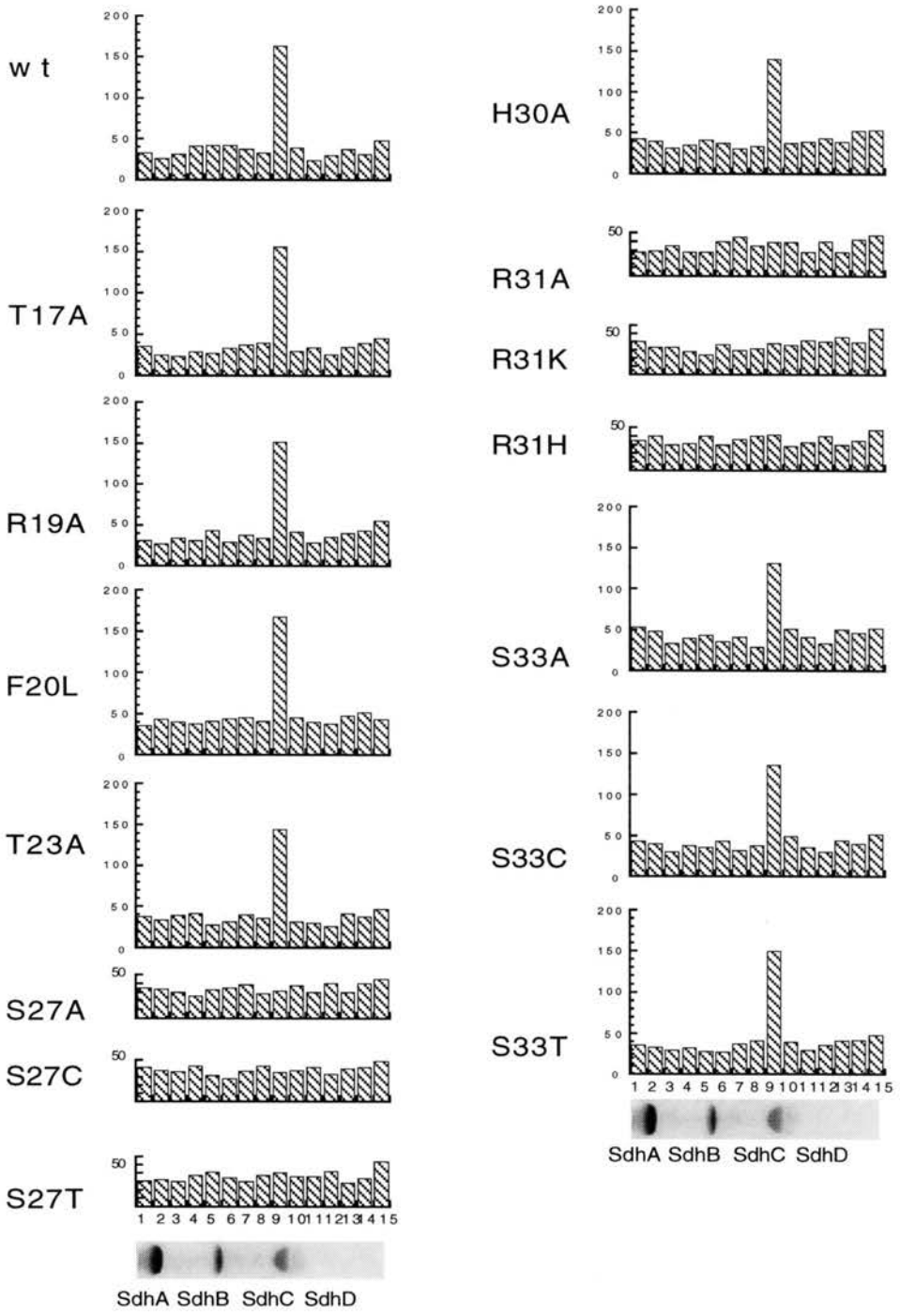


Fig. 14. ^{3}H radioactivity distributions among subunits of mutant succinate-Q reductases. 0.2 ml (2 mg/ml) of each mutant protein is used in the photoaffinity labeling experiment as described in Chapter III, page 51.

distributions among subunits of succinate-Q reductases. Mutants T17A, R19A, F20L, T23A, and H30A succinate-Q reductases have similar azido-Q incorporations as wild type, indicating that these mutations do not affect Q-binding.

Ser-27 and Arg-31 of SdhC are conserved residues in all the type-C membrane anchors of SQR (39). Replacing Ser-27 or Arg-31 with alanine yields cells unable to grow aerobically in M9/succinate medium, indicating that these two are critical residues for succinate-Q reductase. The S27A- and R31A-substituted succinate-Q reductase obtained from their respective mutant cells grown aerobically in LB medium has little electron transfer activity. The involvement of Ser-27 and Arg-31 in the Q-binding of succinate-Q reductase is further evident from the fact that the purified mutant proteins have no azido-Q labeling after uv illumination (Fig. 14) . Since serine and alanine residues occupy virtually the same volume in protein, the loss in succinate-Q reductase activity observed when alanine replaced serine at position 27 suggests that the hydroxyl moiety of this serine plays some role in maintaining the optimal protein structure/reactivity, most likely by participating in a hydrogen bond at the Q-binding site of SdhC. Arg-31 is also critical for cell growth on succinate and succinate-Q reductase activity. However, since the side chains of arginine and alanine differ signicantly in structure and size, the role of this conserved amino acid residue requires further investigation.

The structural importance of Ser-27 and Arg-31 in Q-binding was further examined by replacing Ser-27 with cysteine and threonine and Arg-31 with lysine and histidine. Similar to S27A substitution, S27C or S27T substitution results in cells unable to support aerobic growth in succinate medium. Succinate-Q reductases derived from these two mutant cells have little electron transfer activity and no azido-Q incorporation (Fig. 14). These results indicate that both the hydroxyl group and the size of the amino acid side chain at position 27 of SdhC are critical for Q-binding in succinate-Q reductase. Since the R31K or R31H substitution also yields cells unable to grow aerobically in the succinate medium and abolishes electron transfer activity and [^{3}H]azido-Q uptake (Fig. 14) in succinate-Q

reductase, the guanidino group, rather than its positive charge, on guanidino group at position 31 of SdhC, is critical for succinate-Q reductase activity. The guanidino group of arginine occupies a much larger space than the primary amine of lysine, extends a longer distance, and may provide more chance for hydrogen bond formation (5 hydrogen atoms verse 2 in lysine). Moreover, it might stabilize Q-binding through π-π interactions between the guanidino group and the benzoquinone ring.

It should be emphasized that the loss of electron transfer activity in S27A, S27C, S27T, R31A, R31K, and R31H-substituted succinate-Q reductases is not due to the failure of association of succinate dehydrogenase to the mutant membrane anchoring subunits (SdhC-SdhD). SDS PAGE of purified mutant succinate-Q reductases shows that the four subunits are present in stoichiometric amounts (Fig. 15). Furthermore, the ratio of FAD to cytochrome b_{556}, and the content of FAD and cytochrome b_{556} in these mutant succinate-Q reductases are the same as those of complement reductase (Table V).

Ser-33 of SdhC is also a conserved residue in all type C membrane anchors of succinate-Q reductase. To see whether this serine has the same structural importance as does serine-27, mutant strains having alanine, cysteine, and threonine replacements at this position were generated and characterized. The S33A, S33C, or S33T mutant cells have retarded aerobic growth rates in succinate medium; about 20%, 20%, and 60% of that of the complement strain, respectively, suggesting that succinate-Q reductases in these mutant cells are partially active. The S33A, S33C, and S33T-substituted succinate-Q reductases have 35%, 44%, and 88% of the succinate-Q reductase activity of complement enzyme, suggesting that the hydroxyl moiety, rather than the size of amino acid side chain, at position 33 of SdhC, is important for succinate-Q reductase activity. The apparent K_ms of these mutants for Q_2 are about the same as that of wild type. The purified mutant proteins have comparable azido-Q uptake as wild type (Fig. 14). Perhaps this serine forms a weak hydrogen bond with quinone and is involved in the protonation of quinone. Thus, the

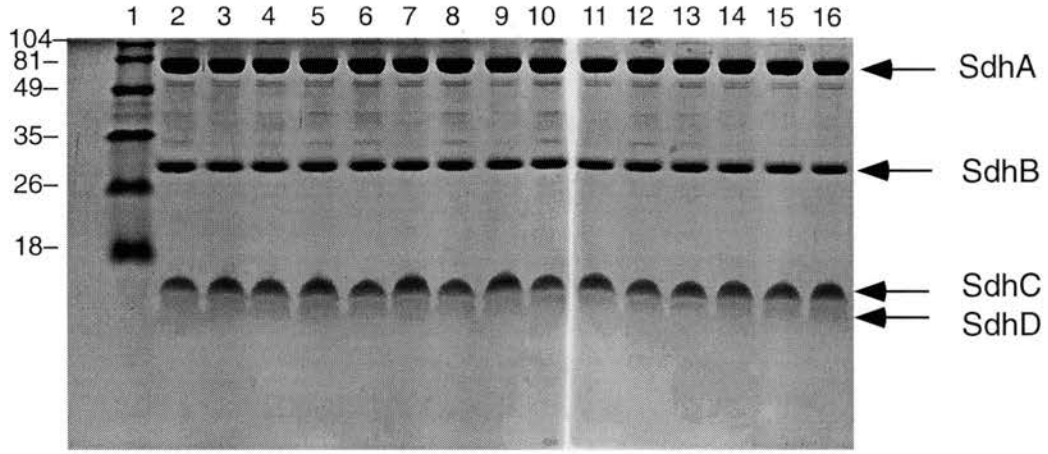


Fig. 15. SDS PAGE of purified mutant succinate-Q reductases. 34 pmol of each mutant SQR (based on cytochrome *b*) is loaded on the gel. Lane 1, molecular weight standard; lane 2, wild type; lane 3, T17A; lane 4, R19A; lane 5, F20L; lane 6, T23A; lane 7, S27A; lane 8, S27C; lane 9, S27T; lane 10, H30A; lane 11, R31A; lane 12, R31K; lane 13, R31H; lane 14, S33A; lane 15, S33C; lane 16, S33T.

mutation of Ser-33 to alanine may greatly reduce enzyme turnover without affecting the affinity for Q.

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VITA

Xudong Yang

Candidate for the Degree of

Doctor of Philosophy

Thesis: STRUCTURE AND FUNCTION STUDIES OF SUCCINATE-UBIQUINONE REDUCTASE FROM *ESCHERICHIA COLI*

Major Field: Biochemistry and Molecular Biology

Biographical:

Personal Data: Born in Siping, Jilin Province, China, on March 9, 1971, the son of Yougeng Yang and Minglan Li.

Education: Graduated from the Northeast Normal University-Affiliated High School, Changchun, Jilin Province, China in May 1989; received Bachelor of Science degree in Biochemistry from Nankai University, Tianjin, China in July 1993. Completed requirements for the Doctor of Philosophy degree with a major in Biochemistry and Molecular Biology at Oklahoma State University in December, 1998.

Professional Memberships: American Association for the Advancement of Science